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Early micro-rheological consequences of single fraction total body low-dose photon irradiation in mice

Kornelia Szluha[a,b], Kornelia Lazanyi[a], Andrea Furka[a], Ferenc Kiss[c], Imre Szabo[a], Eva Pintye[a], Iren Miko[c] and Norbert Nemeth[c,*]

[a]*Department of Radiotherapy, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary,*

[b]*Department of Diagnostic Radiology and Oncotherapy, Semmelweis University, Budapest, Hungary*

[c]*Department of Operative Techniques and Surgical Research, Institute of Surgery, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary*

Abstract. Despite of the studies on widespread biological effects of irradiation, surprisingly only little number of papers can be found dealing with its *in vivo* hemorheological impact. Furthermore, other studies suggested that low-dose irradiation might differ from high-dose in more than linear ways. On Balb/c Jackson female adult mice hematological and hemorheological impacts of total body irradiation were investigated 1 hour following 0.002, 0.005, 0.01, 0.02, 0.05 and 0.1 Gy dose irradiation. In case of 0.01 Gy further groups were analyzed 30 minutes, 2, 4, 6, 24 and 48 h after irradiation. According to the results, it seems that the dose-dependent changes of blood micro-rheological parameters are not linear. The irradiation dose of 0.01 Gy acted as a point of 'inflexion', because by this dose we found the most expressed changes in hematological parameters, as well as in red blood cell aggregation, deformability and osmoscan data. The time-dependent changes showed progressive decrease in pH, rise in lactate concentration, further decrease in erythrocyte aggregation index and deformability, with moderate shifting of the optimal osmolarity point and modulation in membrane stability. As conclusion, low-dose total body irradiation may cause micro-rheological changes, being non-linearly correlated with the irradiation dose.

Keywords: Red blood cell deformability, red blood cell aggregation, osmotic gradient ektacytometry, low-dose irradiation, mice

1. Introduction

Although medical use of radioactive agents is older than a century, it is still an unknown territory in more than a few details. It is widely known, that the ionising effect of radiotherapy causes biological alteration due to its energy being absorbed in tissues. This effect is quantifiable by the means of linear energy transfer (LET) [7]. Irradiations are divided into two main groups based on of their LET value. High LET values belong to alpha radiation, neurons and protons, while low values are characteristic of X-ray, gamma-, beta-, photon and electron radiation [11, 16, 23]. Present article concentrates on the biological effect of irradiation of high LET.

With the spread of modern imaging technologies (CT, PET-CT, etc.) it is inevitable to explore the radiation biological effects of low-dose irradiation. However, literature data are limited in this area.

*Corresponding author: Norbert Nemeth, M.D., Ph.D., Department of Operative Techniques and Surgical Research, Medical and Health Science Centre, University of Debrecen, H-4032 Debrecen, Nagyerdei krt. 98., Hungary. Tel./Fax: +36 52 416 915; E-mail: nemeth@med.unideb.hu.

The biological and neuroimmunological effects of low-dose irradiation are traditionally only estimated deductively from those of high dose, however, recent studies suggest, that low-dose irradiation may differ from high-dose in more than linear ways [6, 8, 11, 27, 48]. Dauer and co-workers even state that low-dose radiation research should be holistic and empirically founded for models, defining the shape of the dose-response relationship to be developed [14].

Despite of the studies on widespread biological effects of irradiation, surprisingly only little number of papers can be found dealing with its *in vivo* hemorheological impact. However, rheological link to radiotherapy seems to be an important aspect. Clinical studies demonstrated the beneficial effects of pentoxifylline (with improving effect on red blood cell deformability) in radiotherapy of breast and lung cancer [20, 36] and experimentally in rhabdomyosarcoma [50] among others. Other clinical hemorheological studies showed such evidences, for example in connection of high-dose brachytherapy in uterine cancer published by von Tempelhoff and co-workers [46]. Some papers deal with the effect of irradiation on red blood cell rheology, mostly in relation of blood storage [19, 38, 45], and sporadically can be found follow-up animal studies [e.g. 49].

Red blood cells' micro-rheological properties, such as deformability and aggregation are determined by numerous factors (cell shape, volume, surface-to-volume ratio, inner viscosity, membrane viscosity, composition of cell surface glycocalyx, fibrinogen concentration, lactate concentration, pH, osmolarity, oxygenation), and play important role in characterization, determination of blood flow characteristic at various levels of the circulation [1, 4, 10, 21, 22, 24, 32, 33, 35, 37, 39, 43, 47].

The effects of irradiation on red blood cells (e.g., change in volume, morphology and membrane integrity, induced oxidative damage, potassium efflux, sodium influx) [7, 17, 19, 30, 31, 38], depending on its dosage and fractionating, thus may have micro-rheological impact, also affecting tissue microcirculation. However, there is still a lack of *in vivo* animal studies in this field.

In this study we hypothesized that low-dose photon irradiation, under 0.1 Gray (Gy), may have detectable micro-rheological effects in mice. We also hypothesized that this impact is not linearly correlated with the dosage and may change in time.

2. Materials and methods

2.1. Experimental animals and groups

The experiments were approved and registered by the University of Debrecen Committee of Animal Research (registration Nr.: 16/2011 UD CAR), in accordance with the Hungarian Animal Protection Act (Law XVIII/1998).

Sixty-five Balb/c Jackson female mice (bodyweight: 20.67 ± 1.68 g; age: 98–105 days) were subjected into two experimental series: in the *Experimental series I* the effects of various doses of irradiation were investigated, in the *Experimental series II* time-dependent effect of one chosen dose of irradiation was analyzed. The mice were randomly divided into the following groups.

2.1.1. Experimental series I

Control group ($n = 5$): no irradiation was made, but all the circumstances of housing, transport, time staying in the irradiation cage were similar to the groups with real irradiation.

Groups of various irradiation doses: 0.002 Gy ($n = 5$), 0.005 Gy ($n = 5$), 0.01 Gy ($n = 5$), 0.02 Gy ($n = 5$), 0.05 Gy ($n = 5$) and 0.1 Gy ($n = 5$). The samplings were performed under general anesthesia exactly 1 hour after irradiation.
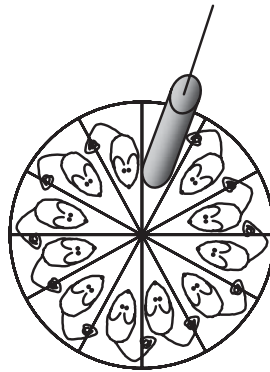
Fig. 1. Schematic drawing of the positioner for homogenous total-body irradiation of mice.

2.1.2. Experimental series II

After the irradiation with 0.01 Gy further time points of investigations were set at 30 minutes ($n = 5$), 2 hours ($n = 5$), 4 hours ($n = 5$), 6 hours ($n = 5$), 24 hours ($n = 5$) and 48 hours ($n = 5$), respectively.

2.2. Technique of irradiation

In a pilot study, we analyzed the body weight-volume relations in the same mice strain. The body volume/body weight ratio was found to be a mean of 1.13 (volume = 22.67 ± 0.81 cm^3, weight = 20 ± 1.26 g; ratio = 1.13 ± 0.03, r^2 = 0.9375; $n = 6$). The irradiation dose fractions were calculated accordingly.

Since the mice were irradiated *in vivo* we had to prepare a special positioner. This device also had a kind of homogenizing effect. Since the mice are relatively small, compared to the size of the irradiation field, the positioner was embedded into a paraffin frame that ensured almost homogeneous dose distribution. Nineteen positioners were placed on a tray that also had a dosimeter on it and were irradiated with the same fraction (Fig. 1). This way, not only the *ex ante* fine-tuning of dosing, but the *ex post* controlling became possible.

Besides creating the special positioner we had to find the suitable technology that can radiate extraordinarily small doses such as 0.002 Gray. Since linear accelerators are not capable of such a fine-tuning and low dose, we decided to use Chisobalt 2B-75 device (Chirama, Czechoslovakia). Chisobalt irradiates with ^{60}Cobalt isotope (^{60}Co T$_{1/2}$ = 5.27 years) at a dose rate of 0.6789 Gy/min, with 1.25 MeV (SSD 100 cm, field size 21×21 cm).

Although ^{60}Co is capable of lower dose, than linear accelerators, shielding of the research objects was also necessary in order to produce the appropriate dose distribution. The thickness of the absorber plastic-sheet was 60 mm. When using Chisobalt the application of PTW Unidos Power Chamber Electrometer (PTW, Germany), a special device became indispensable in order to adjust and measure the exact dose.

2.3. Anesthesia and sampling protocol

Thiopenthal® was used intraperitoneally in 62.5 mg/kg dosage for general anesthesia, under which median thoraco-laparotomy was performed for blood sampling and tissue biopsies. For blood sampling cardiac puncture was made (26 G needle), the samples were taken into tubes with K$_3$-EDTA (1.5 mg/ml) as anticoagulant. Laboratory measurements were completed shortly after sampling, all within 1-2 hours.

After blood sampling biopsies were taken from the heart, lung, liver, spleen, kidney, small bowel and femur for histomorphological examinations. In this paper the laboratory investigations are reported.

2.2. Laboratory measurements

2.2.1. Blood pH and lactate concentration

An ABL555 blood gas analyzer automate (Radiometer Copenhagen, Denmark) was used to determine blood pH and lactate concentration (mmol/l). Following blood sampling, the tests were immediately performed (within 1-2 minutes), without direct contact with air.

2.2.2. Hematological parameters

To determine the general hematological parameters a Sysmex F-800 semi-automated microcell counter (TOA Medical Electronics Co., Japan) was used. Measurements could be carried out within 10-15 minutes after sampling. In this study hemoglobin concentration (Hgb [g/dl]), hematocrit (Hct [%]), mean corpuscular volume (MCV [fl]), mean corpuscular hemoglobin content (MCH [pg]), mean corpuscular hemoglobin concentration (MCHC [g/dl]) and red cell distribution width (RDW-CV% [%]) were analyzed.

2.2.3. Red blood cell deformability

A LoRRca MaxSis Osmoscan device (Mechatronics BV, The Netherlands) was used to measure red blood cell elongation index in the function of shear stress, osmotic gradient ektacytometry parameters and performing membrane stability test.

For *regular red blood cell deformability tests* 5 μl blood sample was required, which was gently mixed in 1 ml of isotonic polyvinyl-pyrrolidone solution (360 kDa PVP in normal phosphate buffered saline; viscosity = 27 mPa.s, osmolarity = 290–300 mOsm/kg; pH ∼ 7.3). The suspension was injected into the bob-cup system of the device, carefully watching not to leave any air bubbles in the measuring chamber. During the measurements, the device generated shear stress (SS) range from 0.3 to 30 Pa, while the laser diffraction pattern was analyzed at set shear stress levels to calculate the elongation index (EI) values. The EI is equal to $(L-W)/(L+W)$, where L is the length and W is the width of the diffractogram. EI increases with red blood cell deformability [2, 18]. The tests were carried out at constant temperature of $37°C$. Since data reduction methods of EI-SS curves are useful tools for comparison [3, 5, 44], Lineweaver-Burk analyses were performed, calculating the maximal elongation index (EI_{max}) and the shear stress at half EI_{max} ($SS_{1/2}$ [Pa]) values, according to the following formula: $1/EI = SS_{1/2}/EI_{max} \times 1/SS + 1/EI_{max}$ [3].

For the *osmotic gradient ektacytometry (osmoscan) measurements* 250 μl blood was needed, which was placed and gently mixed in 5 ml PVP solution, having the same parameters described above. During the measurements, a constant shear stress of 30 Pa was applied to the sample, which is continuously aspirated into the measurement chamber together with changing amount of 0 and 500 mOsmol/kg PVP solutions. The EI values were continuously registered along the osmolarity scale [13, 18]. In this study we analyzed the peak of the elongation index-osmolarity curve, comparing the maximal elongation index values (EI values at the peak of the curve) and the osmolarity at maximal EI ('optimal' osmolarity).

In the *membrane stability test,* we used a continuous shear stress of 100 Pa for 300 seconds. Before and after the mechanical shearing the device measures regular red blood cell deformability tests. We used this function on blood samples of the *Experimental series II.*
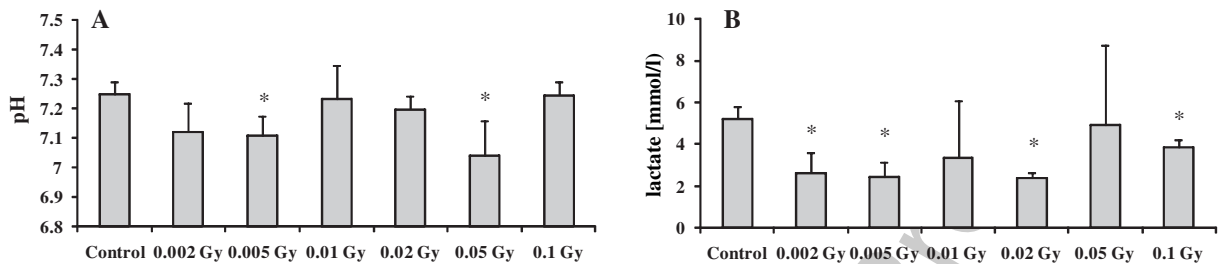
Fig. 2. Changes of blood pH (A) and lactate concentration [mmol/l] (B) 1 hour after various doses of irradiation. means ± S.D.; *$p < 0.05$ vs. Control.

2.2.4. Red blood cell aggregation

Based on light-transmittance method, a Myrenne MA-1 erythrocyte aggregometer (Myrenne GmbH, Germany) was used to determine aggregation index values M (at shear rate of $0 \, s^{-1}$) and M1 (at shear rate of $3 \, s^{-1}$) 5 or 10 seconds after disaggregation. The indices (M 5 s, M1 5 s, M 10 s, M1 10 s) increase with enhanced red blood cell aggregation [2, 18]. The measurements could be carried out within 20–30 minutes after sampling.

2.3. Statistical analyses

Data are expressed as means and standard deviations (S.D.). For comparison of groups with various doses of irradiation, *t*-test or Mann-Whitney rank sum test were used, according to the data distribution. Comparing the changes in time, one way ANOVA on ranks with Bonferroni's method was used. A *p* value of <0.05 was considered as statistically significant.

3. Results

3.1. Effects of various low-dose irradiation (0.002, 0.005, 0.01, 0.02, 0.05 and 0.1 Gy)

3.1.1. Blood pH and lactate concentration

Changes in blood pH or lactate concentration did not reflect obvious relationship to dose-dependency of the irradiation (Fig. 2).

After irradiation at 0.005 and 0.05 Gy we measured significantly lower pH values compared to the Control ($p = 0.009$ and $p = 0.016$, respectively). Lactate concentration decreased in all irradiated groups in various manners. Compared to the Control group significant decrease was found in groups irradiated by 0.002 Gy ($p = 0.006$), 0.005 Gy ($p = 0.001$), 0.02 Gy ($p < 0.001$) and 0.1 Gy ($p = 0.012$).

3.1.2. Hematological data

Selected hematological parameters are summarized in Table 1.

Hematocrit (Hct [%]) showed slight decrease after irradiation at all dose. The most expressed decrease was observed at the 0.01 Gy ($p = 0.01$) and 0.1 Gy doses. If the values were compared to the lowest one, to the 0.01 Gy data, the differences were significant at 0.002 Gy ($p = 0.01$), 0.005 Gy ($p = 0.023$), 0.02 Gy ($p < 0.001$) and 0.05 Gy ($p = 0.007$).

Mean corpuscular volume (MCV [fl]) moderately decreased after 0.002 Gy ($p = 0.003$ vs 0.01 Gy), 0.005 Gy ($p = 0.031$ vs Control; $p < 0.001$ vs 0.01 Gy) and 0.05 Gy irradiation ($p < 0.001$ vs 0.01 Gy).

Table 1

Erythrocyte-related hematological, deformability and osmoscan parameters in blood samples taken from mice 1 hour after various dose of irradiation

Variable	Control	0.002 Gy	0.005 Gy	0.01 Gy	0.02 Gy	0.05 Gy	0.1 Gy
Hct [%]	44.52 ± 5.29	44.39 ± 3.12 #	42.58 ± 1.38 #	37.4 ± 4.95*	44.9 ± 2.34 #	43.42 ± 3.3 #	40.53 ± 7.19
Hgb [g/dl]	11.2 ± 0.81	11.81 ± 0.41	12.04 ± 0.2	10.53 ± 1.47	10.89 ± 0.54	11.64 ± 0.76	11.56 ± 1.46
MCV [fl]	57.33 ± 5.35	54.47 ± 5.79 #	51.47 ± 1.44*	61.03 ± 1.77+	59.27 ± 1.31	53.78 ± 2.68 #	60.56 ± 1.91
RDW-CV% [%]	14 ± 0.22	13.71 ± 1.18	13.25 ± 0.34	14.1 ± 0.45	14.23 ± 0.29	14.07 ± 0.57	13.86 ± 0.31
MCH [pg]	14.45 ± 0.91	14.46 ± 0.65	14.57 ± 0.43 #	15.55 ± 1.28	14.37 ± 0.2 #	14.3 ± 0.4 #	16.75 ± 1.99
MCHC [g/dl]	25.45 ± 3.34	26.68 ± 1.82	28.3 ± 0.67	28.15 ± 4.99	24.26 ± 0.47	26.66 ± 1.37	27.52 ± 3.44
EI_{max}	0.544 ± 0.01	0.538 ± 0.04	0.548 ± 0.02	0.539 ± 0.02	0.557 ± 0.02	0.547 ± 0.01	0.543 ± 0.02
$SS_{1/2}$ [Pa]	1.87 ± 0.3	2.55 ± 0.49*	2.76 ± 0.3*	1.77 ± 0.46	1.85 ± 0.32	1.78 ± 0.12	1.88 ± 0.36
Maximal EI	0.444 ± 0.04	0.487 ± 0.03	0.496 ± 0.01*	0.445 ± 0.08	0.5 ± 0.004*	0.5 ± 0.02*	0.478 ± 0.04
Osmolarity at max. EI [mOsm/kg]	312 ± 20.55	326.2 ± 11.23 #	326.6 ± 9.44 #	293.2 ± 9.98	293.2 ± 7.85	313 ± 6	321 ± 11.66 #

means $\pm$ S.D. EI_{max} = calculated maximal elongation index (EI); $SS_{1/2}$ = calculated shear stress (SS) at half EI_{max} by Lineweaver-Burk analysis; Maximal EI = maximal EI values measured in osmoscan function. *$p < 0.05$ vs. Control; # $p < 0.05$ vs. 0.01 Gy; + $p = 0.053$ vs. Control.
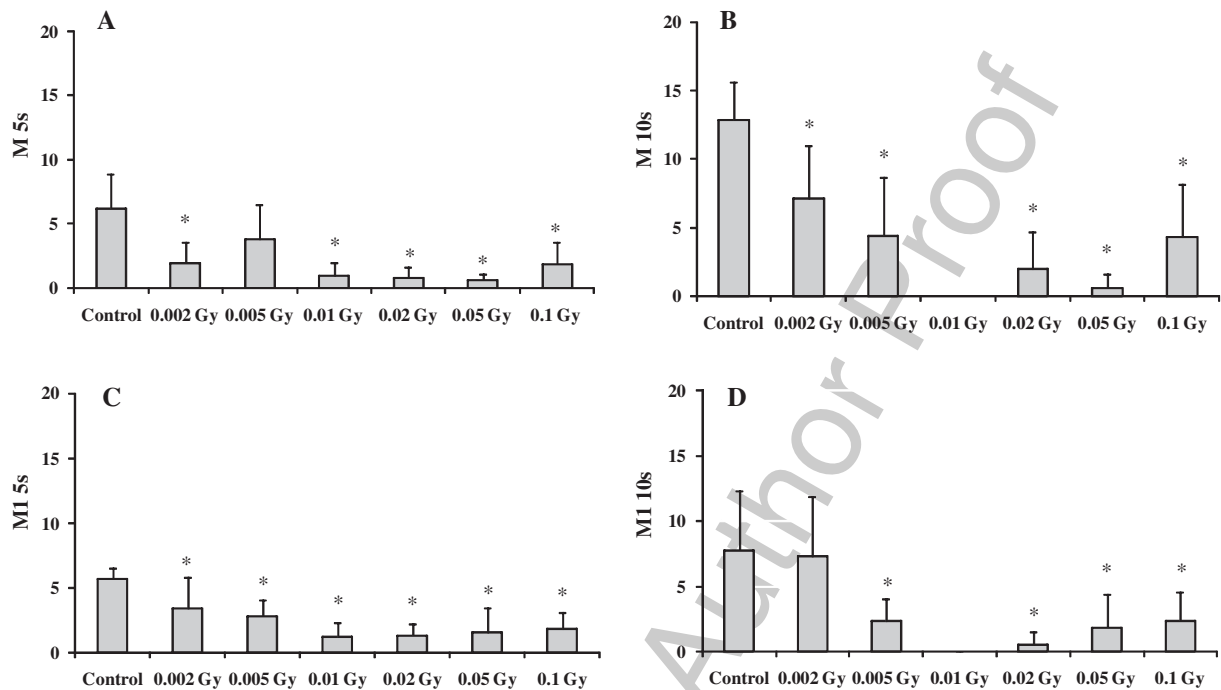
Fig. 3. Changes of aggregation index M 5 s (A), M 10 s (B), M1 5 s (C) and M1 10 s (D) 1 hour after various doses of irradiation. means $\pm$ S.D.; *$p < 0.05$ vs. Control.

MCV values slightly increased in samples taken from groups with irradiation of 0.01 Gy ($p = 0.053$), 0.02 Gy and 0.1 Gy.

Mean corpuscular hemoglobin content (MCH [pg]) showed moderate increase in groups of 0.01 Gy and 0.1 Gy. The other parameters, such as RDW-CVR% or MCHC did not show significant differences.

3.1.3. Red blood cell deformability

Comparative parameters of regular red blood cell deformability and osmotic gradient ektacytometry (osmoscan) measurements are showed in Table 1.

Calculated parameters from the elongation index (EI) - shear stress (SS [Pa]) curves, such as EI_{max} and $SS_{1/2}$ [Pa] did not show obvious relation to the irradiation dose. We did not find any significant difference in EI_{max}, however, $SS_{1/2}$ values were significantly increased compared to the Control only in samples from the groups which were irradiated with 0.002 Gy ($p < 0.001$) or 0.005 Gy ($p < 0.001$).

During osmoscan measurement, we could observe that the maximal elongation index values rather increased in all irradiated groups, but in the smallest manner at 0.01 Gy. The differences versus Control were significant in cases of 0.005 Gy ($p = 0.034$), 0.02 Gy ($p = 0.016$) and 0.05 Gy ($p = 0.046$). However, the osmolarity values at the maximal EI (at the peak of EI-osmolarity curves) were the highest in case of 0.002 Gy ($p < 0.001$ vs. 0.01 Gy), 0.005 Gy ($p < 0.001$ vs. 0.01 Gy) and 0.1 Gy ($p = 0.008$ vs 0.01 Gy).

3.1.4. Red blood cell aggregation

Investigating the aggregation index M and M1 at 5 and 10 seconds, we could observe, that up to 0.01 Gy, the values tended to decrease (even till zero values), than rather increased values were seen, however, all being lower than the Control data. The 0.01 Gy dose seemed like a site of an 'inflexion' point (Fig. 3).
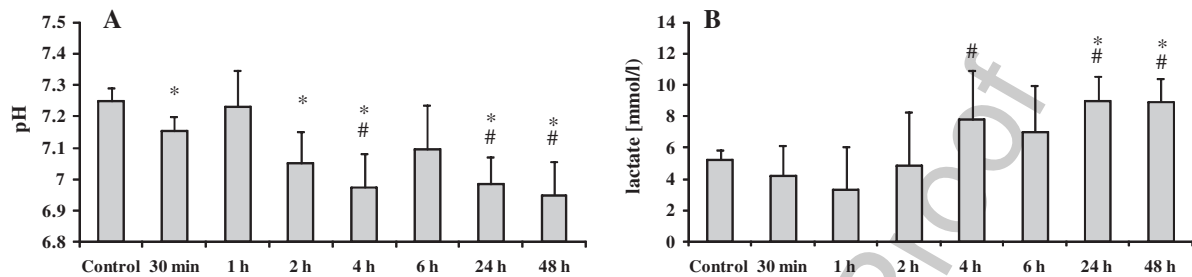
Fig. 4. Changes of blood pH (A) and lactate concentration [mmol/l] (B) in the function of time after 0.01 Gy dose of irradiation. means $\pm$ S.D.; *$p < 0.05$ vs. Control; #$p < 0.05$ vs. 1 h.

M index of 5 seconds (M 5 s) showed significantly lower values at 0.002 Gy ($p < 0.001$), 0.01 Gy ($p < 0.001$), 0.02 Gy ($p < 0.001$), 0.05 Gy ($p < 0.001$) and 0.1 Gy ($p < 0.001$), compared to Control (Fig. 3A).

M index of 10 seconds (M 10 s) also showed significantly lower values at 0.002 Gy ($p < 0.001$), 0.005 Gy ($p < 0.001$), 0.02 Gy ($p < 0.001$), 0.05 Gy ($p < 0.001$) and 0.1 Gy ($p < 0.001$), compared to Control. At 0.01 Gy all measured values were zero (Fig. 3B).

M1 index of 5 seconds (M 5 s) showed the same tendency as M values did. Compared to Control significantly lower values were measured at 0.002 Gy ($p = 0.001$), 0.005 Gy ($p < 0.001$), 0.01 Gy ($p < 0.001$), 0.02 Gy ($p < 0.001$), 0.05 Gy ($p < 0.001$) and 0.1 Gy ($p < 0.001$) (Fig.3 C).

M1 index of 10 seconds (M 10 s) showed significant difference at 0.005 Gy ($p = 0.002$), 0.02 Gy ($p = 0.003$), 0.05 Gy ($p = 0.002$) and 0.1 Gy ($p = 0.003$), compared to Control. Here at 0.01 Gy all measured values were zero, too (Fig. 3D).

3.2. Changes in the function of time after irradiation with 0.01 Gy

3.2.1. Blood pH and lactate concentration

In general, blood pH values showed a continuous declining, while lactate concentration expressed an increasing tendency in the function of time, after irradiation with 0.01 Gy (Fig. 4).

Compared to Control, blood pH values were significantly lower 30 minutes ($p = 0.017$), 2 hours ($p = 0.009$), 4 hours ($p = 0.002$), 24 hours ($p < 0.001$) and 48 hours ($p = 0.001$) after irradiation. Values at 4, 24 and 48 hours were significantly lower versus the 1-hour data, too ($p = 0.006$, $p = 0.005$ and $p = 0.004$, respectively).

Lactate concentration continuously elevated, reaching the highest levels from the 4th hour until the end of the follow-up period. Compared to the Control, we found significant differences at the 24th hour ($p = 0.008$) and by the 48th hour ($p = 0.007$). These 1 and 2-day values were significantly higher compared to the 1-hour data, too ($p = 0.004$ and $p = 0.032$), together with the 4-hour values ($p = 0.043$).

3.2.2. Hematological data

Selected, erythrocyte-related hematological parameters are summarized in Table 2. Hematocrit (Hct [%]) did not show important changes until the 4th hour (except for the 1-hour samples), and over 6 hours the values started to be elevated, being significantly different versus Control (6 h: $p = 0.044$, 24 h: $p = 0.008$ and 48 h: $p = 0.012$) and compared to the 1-hour samples, too ($p < 0.001$ for all). Hemoglobin concentration values (Hgb [g (dl]) slightly increased, while mean corpuscular volume (MCV [fl]) showed

²¹⁶ significant or almost significant decrease compared to Control (30-min: $p = 0.024$, 2 h: $p = 0.055$, 4 h:
²¹⁷ $p = 0.059$, 6 h $p = 0.069$). These values were highly significant versus the data obtained from the 1-hour
²¹⁸ samples ($p < 0.001$ for all). The other parameters, such as RDW-CV%, MCH and MCHC did not show
²¹⁹ important changes.

3.2.3. Red blood cell deformability

²²¹ Comparative data of regular deformability tests and osmotic gradient ektacytometry measurements are
²²² presented in Table 2.

²²³ Except for the data from the 1-hour samples, EI_{max} values showed decrease compared to Control,
²²⁴ reaching significant level at 4 hours ($p = 0.022$), 6 hours ($p = 0.015$), 24 hours ($p = 0.014$) and 48 hours
²²⁵ ($p = 0.002$). Compared to the 1-hour values, EI_{max} values were significantly lowered at 4 hours ($p = 0.05$),
²²⁶ 24 hours ($p = 0.039$) and 48 hours ($p = 0.026$). In turn, $SS_{1/2}$ values were higher versus Control (except
²²⁷ for the 1-hour data), showing significant difference at 30 minutes (versus Control: $p = 0.03$; versus 1 h:
²²⁸ $p = 0.024$), 2 hours (versus Control: $p < 0.001$; versus 1 h: $p < 0.001$) and 4 hours (versus Control: $p = 0.001$;
²²⁹ versus 1 h: $p = 0.002$).

²³⁰ During osmotic gradient ektacytometry measurement, the maximal EI values were increased compared
²³¹ to the Control, being significant at 30 minutes ($p = 0.032$), 2 hours ($p = 0.03$), 6 hours ($p = 0.008$) and the
²³² same for 48 hours ($p = 0.008$). The osmolarity values at the maximal EI were increased versus Control (at
²³³ 6 h: $p = 0.023$; 48 h: $p = 0.003$) and versus the 1-hour data (30 min: $p = 0.008$; 2 h: $p = 0.001$; 4 h: $p = 0.026$;
²³⁴ 6 h: $p < 0.001$; 24 h: $p = 0.004$; 48 h: $p < 0.001$).

²³⁵ Investigating membrane stability, we used shear stress of 100 Pa for 5 minutes. The elongation index (EI)
²³⁶ - shear stress (SS) curves of the deformability measurements completed before and after the mechanical
²³⁷ shearing of samples, taken at various time points after irradiation, and results are plotted on Fig. 6.
²³⁸ Comparative data on EI-SS curves are summarized in Table 3. As expected, after the given mechanical
²³⁹ stress, deformability of red blood cells decreased, but in different manner in the samples taken at various
²⁴⁰ times after irradiation. The smallest difference was found at the 2-hour, 4-hour and 6-hour samples. Table 4
²⁴¹ shows the ratios of the values measured after versus before mechanical stress. In the function of shear
²⁴² stress, values over 1 Pa showed similar magnitude. Data at 3 Pa were selected for statistical comparison.
²⁴³ Compared to the 30-minute data, values were significantly higher at 2 hours ($p < 0.001$), 4 hours ($p = 0.004$)
²⁴⁴ and 6 hours ($p = 0.042$), when the before-after differences were the smallest in the original EI-SS curves,
²⁴⁵ too. Values at 6 hours showed difference compared to the 2-hour values, too ($p = 0.039$).

3.2.4. Red blood cell aggregation

²⁴⁷ In the following-up study part, a moderate decreasing tendency was observed in all the four aggregation
²⁴⁸ index parameters, except for the values from the 1-hour samples (Fig. 5).

²⁴⁹ Compared to the Control group, M index of 5 seconds (M 5 s) showed significantly lower values only
²⁵⁰ at 6 hours ($p < 0.001$), 24 hours ($p = 0.002$) and 48 hours ($p < 0.001$). While compared to the 1-hour data,
²⁵¹ values were significantly different at 30 minutes ($p < 0.001$), 2 hours ($p < 0.001$), 4 hours ($p < 0.001$), 6
²⁵² hours ($p = 0.002$) and 24 hours ($p < 0.001$) (Fig. 5A).

²⁵³ M index of 10 seconds (M 10 s) showed significantly lower values only at 6 hours ($p < 0.001$) and 24
²⁵⁴ hours ($p < 0.001$), compared to Control (Fig. 5B).

²⁵⁵ Compared to the Control groups, all M1 index of 5 seconds (M 5 s) values were significantly lower
²⁵⁶ over the follow-up period ($p < 0.001$ for all, except for the 24-hour: $p = 0.002$). Compared to the 1-hour
²⁵⁷ data, the differences were significant at 30 minutes ($p < 0.001$), 2 hours ($p < 0.001$), 4 hours ($p = 0.003$),
²⁵⁸ 6 hours ($p = 0.002$) and 24 hours ($p < 0.001$) (Fig. 5C).

Table 2

Time-dependent changes of erythrocyte-related hematological, deformability and osmoscan parameters in blood samples taken from mice irradiated with 0.01 Gy (Control and 1 h values are transferred from Table 1)

Variable	Control	30 min	1 h	2 h	4 h	6 h	24 h	48 h
Hct [%]	44.52 ± 5.29	44.36 ± 2.67 #	37.4 ± 4.95*	44.27 ± 4.95 #	44.44 ± 3.23 #	49.42 ± 4.25*#	50.92 ± 4.25*#	52.01 ± 6.6*#
Hgb [g/dl]	11.2 ± 0.81	12.08 ± 0.48*#	10.53 ± 1.47	12.16 ± 0.27*#	12.33 ± 0.82*#	13.46 ± 0.5*#	13.45 ± 0.42*#	13.44 ± 0.42*#
MCV [fl]	57.33 ± 5.35	52.89 ± 1.98*#	61.03 ± 1.77	51.82 ± 1.14 #	51.75 ± 1.01 #	53.3 ± 3.78 #	55.1 ± 3.85 #	54.8 ± 3.95 #
RDW-CV% [%]	14 ± 0.22	14.21 ± 0.79	14.1 ± 0.45	13.64 ± 0.34	13.79 ± 0.34	14.71 ± 1.05	14.26 ± 0.66	14.46 ± 0.38
MCH [pg]	14.45 ± 0.91	14.36 ± 0.51	15.55 ± 1.28	14.15 ± 0.52	14.36 ± 0.27	14.56 ± 0.491	14.73 ± 0.78	14.22 ± 0.59
MCHC [g/dl]	25.45 ± 3.34	27.31 ± 1.71	28.15 ± 4.99	27.5 ± 0.86	27.76 ± 0.52	27.43 ± 2.41	26.81 ± 2.17	26.13 ± 2.55
EI_{max}	0.544 ± 0.01	0.502 ± 0.02	0.539 ± 0.02	0.527 ± 0.04	0.494 ± 0.06*#	0.508 ± 0.03*	0.492 ± 0.05*#	0.51 ± 0.02*#
$SS_{1/2}$ [Pa]	1.87 ± 0.3	2.28 ± 0.46*#	1.77 ± 0.46	2.73 ± 0.49*#	2.72 ± 0.62*#	2.12 ± 0.6	2.1 ± 0.49	2.01 ± 0.56
Maximal EI	0.444 ± 0.04	0.497 ± 0.005*	0.445 ± 0.08	0.504 ± 0.007*	0.465 ± 0.04	0.504 ± 0.002*	0.471 ± 0.04	0.505 ± 0.002*
Osmolarity at max. EI [mOsm/kg]	312 ± 20.55	334.8 ± 8.64 #	293.2 ± 9.98	332.5 ± 12.07 #	326.3 ± 22.81#	346 ± 17.5*#	333 ± 19.35 #	360.2 ± 14.7*#

means $\pm$ S.D. EI_{max} = calculated maximal elongation index (EI); $SS_{1/2}$ = calculated shear stress (SS) at half EI_{max} by Lineweaver-Burk analysis; Maximal EI = maximal EI values measured in osmoscan function. *$p < 0.05$ vs. Control; # $p < 0.05$ vs. 1 h.

Table 3

Changes of red blood cell deformability parameters during membrane stability test, presenting values before and after the mechanical stress (shearing at 100 Pa for 300 sec)

Variable		30 min	2 h	4 h	6 h	24 h	48 h
EI at 3 Pa	before	0.238 ± 0.01	0.277 ± 0.02	0.242 ± 0.03	0.32 ± 0.02	0.311 ± 0.02	0.33 ± 0.01
	after	$0.137 \pm 0.01*$	$0.23 \pm 0.01*$	$0.211 \pm 0.04*$	$0.237 \pm 0.02*$	$0.202 \pm 0.02*$	$0.223 \pm 0.02*$
EI_{max}	before	0.493 ± 0.02	0.494 ± 0.02	0.489 ± 0.02	0.535 ± 0.05	0.499 ± 0.03	0.502 ± 0.01
	after	$0.407 \pm 0.01*$	$0.438 \pm 0.04*$	$0.408 \pm 0.06*$	$0.442 \pm 0.02*$	$0.402 \pm 0.02*$	$0.434 \pm 0.03*$
$SS_{1/2}$ [Pa]	before	1.82 ± 0.34	2.43 ± 0.24	2.85 ± 1.04	1.34 ± 0.66	1.58 ± 0.46	1.32 ± 0.29
	after	$7.03 \pm 1.01*$	$4.13 \pm 0.57*$	$4.18 \pm 1.59*$	$5.79 \pm 1.24*$	$6.73 \pm 1.3*$	$6.61 \pm 1.31*$

means $\pm$ S.D. EI = elongation index; EI_{max} = calculated maximal EI; $SS_{1/2}$ = calculated shear stress (SS) at half EI_{max} by Lineweaver-Burk analysis $*p < 0.05$ vs. before.
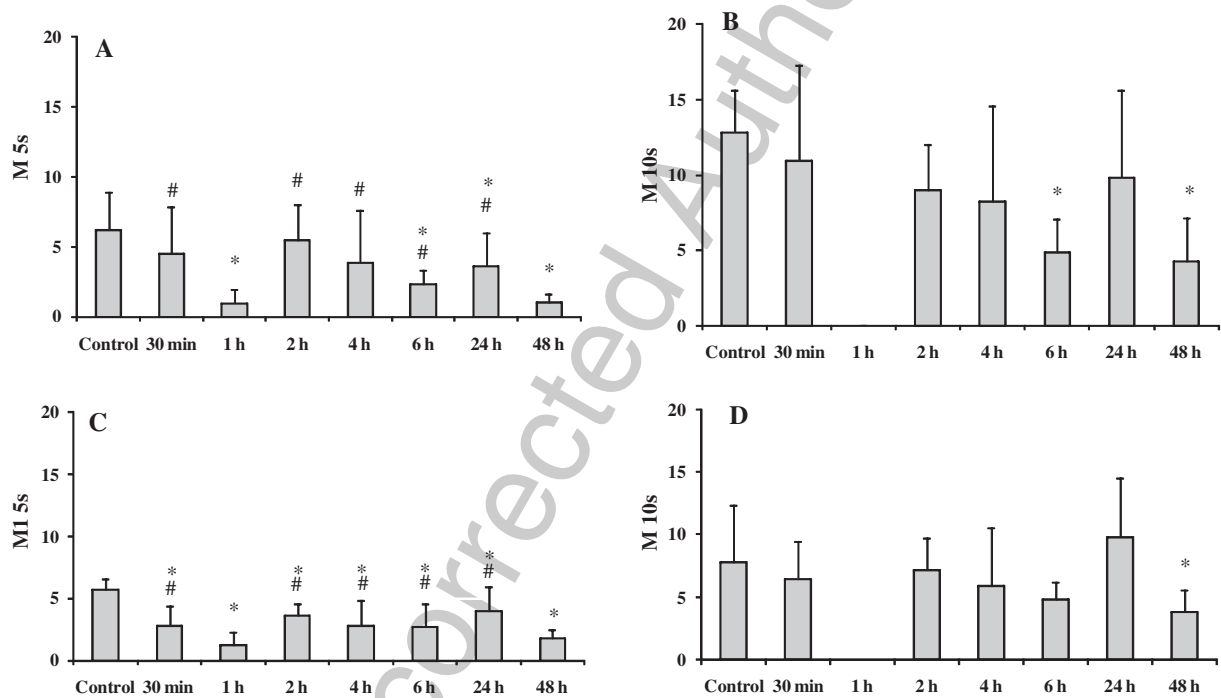

Fig. 5. Changes of aggregation index M 5 s (A), M 10 s (B), M1 5 s (C) and M1 10 s (D) in the function of time after 0.01 Gy dose of irradiation. means $\pm$ S.D.; $*p < 0.05$ vs. Control; $\#p < 0.05$ vs. 1 h.

M1 index of 10 seconds (M 10 s) showed significantly lower values versus Control at 24 hours ($p = 0.018$) (Fig. 5D).

4. Discussion and conclusions

The anti-inflammatory and sterilisation effects of irradiation are well known and clinically widespread for a long time [7]. It is used in case of traumas, viral inflammations, leukaemia and even in case of
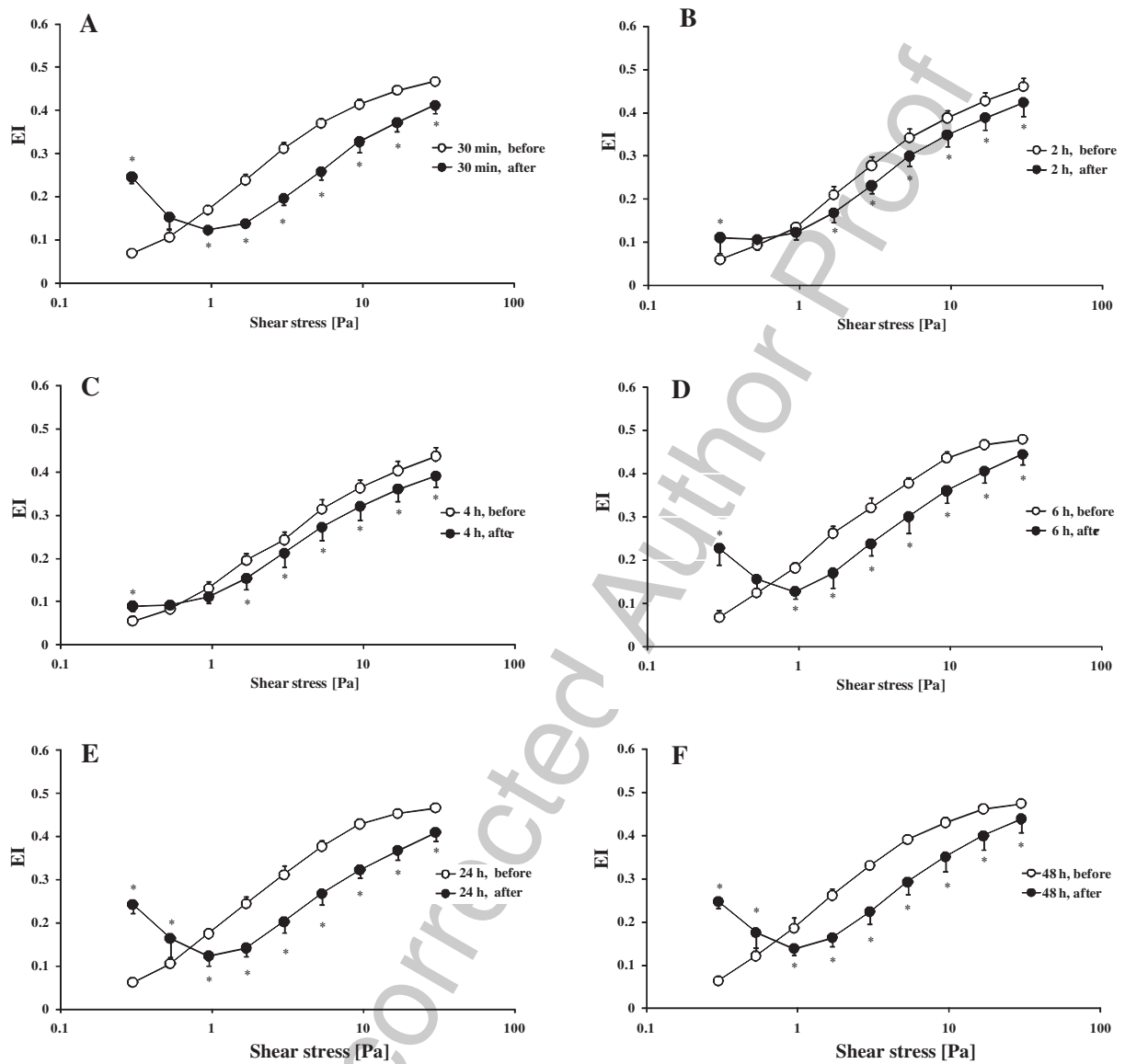
Fig. 6. Elongation index (EI) - shear stress (SS) curves of the deformability measurements completed before (white) and after (black) the mechanical shearing (100 Pa for 5 minutes) of samples taken at various time points (A: 30 minutes, B: 2 hours, C: 4 hours, D: 6 hours, E: 24 hours, F: 48 hours) after irradiation at 0.01 Gy. means ± S.D.; *$p < 0.05$ vs. before.

transfused blood to prevent graft versus host disease [7, 9, 30, 31, 38]. Nevertheless, the optimisation of dosing is in its inauguration stage.

Selim et al. analyzed the effects of 25, 50 and 100 Gy gamma-rays on stored red blood cells using alpha-lipoic acid, in order to help preserving cell viability and structural integrity of the cell membrane, reducing the effect of oxidative damage [15, 40, 41]. The dose of 25 Gy was found to be safe related to erythrocyte integrity. However, this dose may cause significantly increased mean cell volume, as showed

Table 4

Ratios (after/before) calculated from elongation index values at different shear stress values measured before and after mechanical shearing at 100 Pa for 300 sec

Timepoint	Shear stress [Pa]						
	0.95	1.69	3	5.33	9.49	16.87	30
30 min	0.72 ± 0.04	0.57 ± 0.03	0.63 ± 0.07	0.69 ± 0.06	0.79 ± 0.03	0.83 ± 0.03	0.88 ± 0.02
2 h	0.91 ± 0.13	0.8 ± 0.07	$0.83 \pm 0.04*$	0.87 ± 0.01	0.89 ± 0.03	0.9 ± 0.02	0.91 ± 0.03
4 h	0.85 ± 0.1	0.78 ± 0.07	$0.86 \pm 0.11*$	0.86 ± 0.08	0.87 ± 0.06	0.89 ± 0.07	0.89 ± 0.07
6 h	0.7 ± 0.11	0.65 ± 0.12	$0.74 \pm 0.07*\#$	0.79 ± 0.09	0.82 ± 0.09	0.86 ± 0.04	0.92 ± 0.04
24 h	0.71 ± 0.14	0.57 ± 0.04	0.65 ± 0.04	0.71 ± 0.05	0.75 ± 0.04	0.81 ± 0.03	0.87 ± 0.03
48 h	0.75 ± 0.07	0.62 ± 0.04	0.67 ± 0.07	0.74 ± 0.06	0.81 ± 0.05	0.86 ± 0.05	0.92 ± 0.05

means $\pm$ S.D. $*p < 0.05$ vs. 30 min, $\# p < 0.05$ vs. 2 h.

by Kim et al. [19]. Cicha et al. also demonstrated that erythrocyte deformability impairs in exposure to a 35 Gy gamma irradiation [12].

Mihaescu et al. used 20 Gy in mice to investigate the prothrombotic impact in the microcirculation of the large bowel, which was partly mediated by P-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) [28]. Inhibition or lack of P-selectin or PSGL-1 may reduce the thrombotic events in blood vessels [29].

Micke et al. investigated the effects of low-dose irradiation on neutrophil granulocytes of human donors [27], on the other hand, Reverberi, Govoni and Verenini reported the effects on human red blood cells [38]. Serhatlioglu et al. concentrated on the long term effects of recurrent low-dose irradiations on CD4+ T-lymphocyte ratio and serum total IgA, IgG, IgM and C3, C4 levels with the help of volunteering radiologists and those who have never been directly or indirectly irradiated so far [42].

However, immediate (short-term) effects of irradiation of blood of living organisms are near to nonexistent. Maks et al. analyzed the white blood cell counts of mice after proton and gamma irradiation up to 2 Gy by dose rates of 0.5 Gy/min or 0.5 Gy/h. They found a dose-dependent decrease of total leukocyte count [23]. Bogdandi et al. used as low as 0.01-0.1 Gy dose total-body irradiation in mice, demonstrating dose-dependent differences up to 2 Gy in apoptotic processes in various immune cells [8]. Still, *in vitro* research data on low-dose photon irradiation of the blood of living organism are abundant.

Present paper endeavours to narrow this gap in knowledge by supplying data on short-term red blood cell alteration of living mice after low-dose photon irradiation.

According to the results, it seems that the dose-dependent changes of blood micro-rheological parameters are not linear. The irradiation dose of 0.01 Gy acted as a point of 'inflexion', because by this dose we found the most expressed changes in the hematological parameters, as well as in the red blood cell aggregation, deformability and osmoscan data. This was the reason why we have chosen this dose for the second part of the study, where the time-dependent changes were analyzed further. In the literature, we did not find other study using these very low doses of irradiation together investigation of micro-rheological parameters.

Wen et al. also used ^{60}Co irradiation, but in higher dose, for studying hemorheological changes in an animal model. They found that 7 Gy total-body irradiation caused long-lasting decrease of hematocrit (over 10 days, and slow normalization over 30 days) in rabbits, accompanied by decreased deformability index [49].

Red blood cell deformability is determined by cell volume, surface-volume ratio, cell morphology, inner viscosity (hemoglobin content), and membrane viscosity [1, 2, 24, 35]. Red blood cell aggregation

is determined or influenced also by the cell-morphology, but by the composition of the surface glycocalyx, as well as the plasmatic factors, such as the fibrinogen concentration together with the shearing forces [4, 35, 37]. Important influencing factors are the oxygenation, the temperature, the pH and the osmolarity of the micro-environment of the red blood cells [1, 4, 33, 34, 37]. Irradiation of various origins may interact with these factors at numerous points, by influencing the cell volume, the membrane integrity by oxidative damage and structural changes [9, 12, 17, 26, 30, 38]. Since red blood cell deformability plays important role in determining blood flow and microcirculation [1, 10, 22, 43], these changes may cause further alteration in tissue perfusion.

However, the background, dose- and time-dependency of the direction of the changes, like improving or impairing micro-rheological variables are still unclear. Does decreased aggregation mean a rheological improvement after irradiation? Or does it reflect such changes in cellular factors that may reduce the aggregation ability but together with a relatively stable deformability and with shifting of the optimal osmolarity (osmolarity at maximal EI)? Too composite questions so far.

Using laser irradiation (632.8 and 532 nm) Mi et al. reported reduced blood viscosity, and red blood cell deformability could be improved in human blood [25]. They hypothesized that the 'mobilization' of membrane-linked hemoglobin might cause this enhancing effect, which has been demonstrated in their later porcine study [26].

It seems that together with micro-rheological changes, irradiation may cause a complex impact on the blood organ with labyrinthine interactions.

Therefore, it would be also interesting to see more exact evidences for a kind of inflexion point of changes in the function of dose and time. For revealing the background and find evidences, further studies are needed in the future using hemorheological, hematological, hemostaseological, immunological and histomorphological as well as ultrastructural complex investigations.

Acknowledgments

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