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Effects of low doses of gamma rays on the stability of normal and diabetic erythrocytes

Magdalena Kaczmarska[1], Zofia Kopyściańska[1], Maria Fornal[2], Tomasz Grodzicki[2], Krzysztof Matlak[1], Józef Korecki[1] and Květoslava Burda[1]✉

[1]AGH University of Science and Technology, Faculty of Physics and Applied Computer Science, Kraków, Poland; [2]Collegium Medicum, Jagiellonian University, Department of Internal Medicine and Gerontology, Kraków, Poland

We studied the influence of low doses of γ radiation (from 0.04 to 1.8 mGy) on the stability of human red blood cells (RBC) from healthy donors and diabetic patients using absorption spectroscopy. Because of the alteration of many enzymatic pathways in diabetic RBCs resulting in strong modification of the lipid and protein membrane components one could expect that the ionizing γ-radiation should influence the stability of the healthy and diabetic cells in a different way. Indeed, distinct discontinuities and monotonic changes of hemolysis detected in the healthy and diabetic RBCs suggest that various enzymatic and chemical processes are activated in these membranes by γ radiation. Mössbauer measurements showed that only the highest applied dose of γ radiation caused modification of hemoglobin in both types of RBCs.

Keywords: erythrocytes; diabetes; hemoglobin; γ rays; hemolysis; absorption spectroscopy; Mössbauer spectroscopy

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INTRODUCTION

Highly reactive oxygen species (ROS) are natural by-products of oxygen metabolism in living organisms. At physiological concentrations they play important protection and signaling roles but at higher concentrations they are harmful (Meyers**,** 1995). Under normal circumstances, cells are able to defend themselves against ROS damage. Biological effects of these highly reactive compounds are controlled *in vivo* by a wide spectrum of antioxidant mechanisms, as for example by the enzymes superoxide dismutases (SOD), glutathione peroxidase (GPx), catalases (CAT), peroxiredoxin, and also by small antioxidant molecules as carotenoids, tocopherols, ascorbic acid and glutathione (Meister, 1994; Bannister *et al.*, 1987; Johnson *et al.*, 2005; Chelikani *et al.*, 2004; Padayatty *et al.*, 2003; Bagchi *et al.*, 1998; Preedy *et al.*, 2007; Sies *et al.*, 1996). Oxidative stress resulting from increased free-radical production or defects in the antioxidant defenses is implicated in the athogenesis of numerous diseases (Harman, 1992). At the molecular level increased peroxidation of lipids and proteins and even damage of DNA are observed in such cases.

ROS are also generated by exogenous sources, for example ionizing radiation. All living organisms are continuously exposed to background ionizing radiation coming from natural sources such as radioelements in the soil or cosmic radiation as well as man-made ionizing radiation (medical procedures, consumer products, for example food). It has been estimated that this background ionizing radiation amounts to about 2.5 mSv per year at the sea level but at some areas this dose rate is enhanced by a factor of 3 to 10 (high-level natural background radiation) (Lowenthal *et al.*, 1997; Tsun-Yee Chiu *et al.*, 1997). It is well known that high doses of ionizing radiation (exceeding 200 mSv, which corresponds to 200 mGy for γ-radiation) result in overproduction of oxygen-derived free radicals (Zaider *et al.*, 1994; Trosco *et al.*, 1997), which cause peroxidation of membrane proteins and lipids being the first targets of the ROS destructive action (Köteles, 1982). Further products of peroxidation are mutagenic and carcinogenic, for example the reactive malondialdehyde (Dainiak *et al.*, 1995; Dainiak, 1997; Marnett, 1999; Marnett *et al.*, 1994). However, lipid peroxidation is also produced at low doses of ionizing radiation, even close to the normal background one, independently of its type (X-ray or γ-radiation), (Petkau, 1971; Petkau 1972; Riley, 1994). Once initiated in the membrane, the damaging chain reactions propagate by themselves.

This work intends to study the radiation effects of low doses of γ-radiation (from 0.04 mGy to 1.8 mGy) on the red blood cell (RBC) stability. The dose rate of 0.04 mGy /min is only 8000 times higher than that from the background radiation which is about 5 nGy/min. Usually the dose rates investigated exceed this value by tens of orders of magnitude (Gwoździński, 1991; Iyer *et al.*, 2002; Szweda-Lewandowska *et al.*, 2003; Kokosz *et al.*, 2006). γ-Radiation doses of 500–1000 Gy induced alterations of the RBC membrane at the level of lipid bilayer and skeleton proteins influencing in this way the membrane permeability and cell stability as well as hemoglobin states inside the cells (Shapiro *et al.*, 1968; Gwoździński, 1991; Iyer *et al.*, 2002). Much less is known about the cellular response to low doses of ionizing radiation such as those typical for medical diagnostic procedures, normal occupational exposures or cosmic-ray exposures at flight altitudes, and there are only a few reports on their action on RBCs. For example, γ-irradiation (or X-irradiation) of blood components with doses of 25–50 Gy is used to prevent posttransfusion-associated graft-*versus*-host dis-

✉e-mail: burda@novell.ftj.agh.edu.pl

Abbreviations: CAT, catalse; GPx, glutathione peroxidase; Hb, hemoglobin; OxyHb, oxygenated hemoglobin; MetHb, methemoglobin; RBC, red blood cell; SOD, superoxide dismutases; TA-GVHD, posttransfusion-associated graft-*versus*-host disease.

ease, TA-GVHD, (Treleaven *et al.*, 2011). However, this process has been demonstrated to cause an increase in the permeability of the RBC membrane to potassium, sodium, hemoglobin and lactate dehydrogenase (LDH) (Hirayama *et al.*, 2005; Agarwal *et al.*, 2005; Zimmermann *et al.*, 2011; Bashir *et al.*, 2011). It was also shown that exposure to γ-rays with doses of 25 Gy significantly increased the RBC volume and lipid peroxidation. The deformability of the irradiated RBCs was significantly lower than that of non-irradiated cells (Kim *et al.*, 2008). The decreased deformability of γ-ray-irradiated RBCs was independently confirmed by Cicha *et al.* (2000) but they did not observe an enhancement of lipid peroxidation, protein aggregation of the RBC membrane, or echinocytosis. Some of the contradictory results may originate from the differences in the medium used. Exposure of the whole body to single doses of γ-rays of 1 to 9 Gy revealed an increase of blood viscosity as a function of the dose from 1 Gy up to 7 Gy but for 9 Gy a decrease of viscosity was detected. In addition, the RBC membrane surface charge density decreased after irradiation by the lowest dose and continued to decrease with the increased dose, which was demonstrated by measuring the relative permittivity and relaxation time at the low frequency range in RBC membrane surface (Selim *et al.*, 2009). Investigations of the biochemical and biophysical properties of human erythrocyte membranes after exposure to relatively low doses of γ rays (2, 4 and 8 Gy and the rate dose of 0.5 Gy/min), performed by Benderitter *et al.* (2003), showed that the lipid peroxidation increased with an increased postirradiation time (from 3 to 72 h), whereas antioxidant activities of catalase and glutathione peroxidase decreased after irradiation and the change was more significant with increasing dose. The SOD activity had a similar behavior as catalase and glutathione peroxidase 3 h after exposure but at 72 h after exposure it increased with the increased dose of radiation. Those observations were made in relation to corresponding controls but it is interesting to mention that the SOD activity decreased significantly in the control sample after 72 h of incubation in comparison with the same sample after 3h whereas the activities of catalase and glutathione peroxidase were comparable for both periods of incubation. In the case of skeleton proteins, spectrin and actin oxidation was highest for the lowest dose applied, i.e. 2 Gy for 3 h after irradiation. Then, actin oxidation decreased clearly with the increasing dose but spectrin oxidation stayed the same. All these modifications together with the detected increased content of the saturated phosphatidylethanolamine (PE) fatty acid and decreased content of the (n-3) and (n-6) series of PE fatty acids results in an increased fluidity of the membrane lipid compartment but increased rigidity of the lipid-protein membrane interface.

Basic research data and human epidemiological data show that cellular responses to low absorbed doses of ionizing radiation cannot be predicted by extrapolating from the responses to high doses. Numerous examples of 'radiation hormesis' and 'radioadaptive response' can be found in the literature (Meyer *et al.*, 1998; Radivoyevitch *et al.*, 2002; Cohen, 2008; Iyer & Lehnert, 2002; Feinendegen, 2005; Chen *et al.*, 2007). Depending on the type of adaptive protection in a given cell system, except for apoptosis and terminal cell differentiation, the adaptive response has a maximum for doses from 5 mGy to 200 mGy in most mammalian cells (Meyer *et al.*, 1998). Recently, it has been found that the biological effects of low and high LET (linear energy transfer) radiations at low doses are very complex because of a newly observed phenomenon called the "bystander effect", when non-irradiated cells exhibit responses like those of the neighbouring irradiated cells (Azzam *et al.*, 1998; 2001; Mothersill & Seymour, 2001; Zhou *et al.*, 2001; Nagasawa & Little, 2001; Sgouros *et al.*, 2007; Iyer & Lehnert, 2002; Baskar, 2010). Positive biological effects of acute low doses (0.01–0.05 Gy) of γ-radiation on the erythrocyte membrane were demonstrated by Mahmoud *et al.* (2011). The same authors showed that higher doses of 0.2 and 0.3 Gy caused aggregation of proteins and changes of organization of phospholipids of the RBC membrane. However, within the framework of this study we concentrated on probing the effects of much lower doses of γ-radiation, from 0.04 mGy to 1.8 mGy, which are at least 1–3 orders of magnitude lower than those at which hormesis phenomena have been reported. Therefore all effects which are described in this paper could be related to the Petkau effect taking place when the propagation of free radicals is enhanced. It has been observed that μGy doses can cause serious oxidative stress in living organisms and in addition at lower dose rates result in more severe damage to lipids and proteins, especially riche in –SH groups (Petkau, 1972; Graeub, 1994).

Taken together, these findings reinforce our understanding that the cell membrane is a significant biological target of radiation. Thus the role of the biological membrane in the expression and course of cell damage after radiation exposure must be considered. Both OH^- and $O_2^{\bullet -}$ radicals play a role in the initiation of lipid peroxidation in human erythrocytes (Purohit *et al.,* 1980a; 1980b; Köteles, 1982). Because of the Petkau effect one may expect that under our experimental conditions hemolysis of irradiated RBCs should be observed and it should depend on the state of the RBC membrane. Although RBC is not a very radiosensitive cell, it is a suitable candidate for monitoring the radiation effects because: (i) it is a representative sample for the whole body exposure, (ii) it is easy to obtain RBC with an intact membrane, (iii) the RBC membrane has a well known structure (Pasini *et al.*, 2006) and (iv) RBC is very often used to monitor the stage of development of many diseases, for example of diabetes (Baynes *et al.,* 1999). Therefore in our studies we chose RBCs isolated from the blood of healthy donors and diabetic patients. Diabetic disease is related to the metabolic perturbation or abnormal blood glucose homeostasis and chronic hyperglycemia (Ha *et al.,* 1999; Baynes *et al.,* 1999). In diabetic cases, modifications of RBC membrane properties and of RBC inner homeostasis have been reported (Ha *et al.,* 1999; Nagamatsu *et al.,* 1986; Waczulíková *et al.,* 2000; Adak *et al.,* 2008). These changes result mainly from an increased level of lipid and protein peroxidation as well as of glycooxidation products in diabetic RBCs in comparison to control RBCs (Resmi *et al.,* 2001). There is even a glycooxidation hypothesis assuming that glycooxidation is responsible for the permanent, cumulative oxidative damage to long-lived proteins in aging and in diabetes (Baynes, 1991). In addition, it has been proven experimentally that many enzymatic reactions defending the erythrocyte membranes against ROS are altered in diabetic RBC membranes (Ha *et al.,* 1999; Uzel *et al.*, 1987; Stevens *et al.*, 2000; Murakami *et al.*, 1989; Yoshida *et al.*, 1995; Thornalley *et al.*, 1996; Lang *et al.,* 1966; Venerando *et al.,* 2002). Thus, irradiation of healthy and diabetic RBCs applying such a low rate of γ-radiation as 0.04 mGy/

min for a short time only (from 1 to 45 min) allows us to follow differences in their sensitivity to γ-radiation. One should expect differences in their response to the oxidative stress induced by ionizing radiation knowing that the properties of diabetic RBCs vary from those of healthy RBCs. The percentage of hemolysis of RBCs treated with γ-rays was estimated from absorption measurements. Mössbauer spectroscopy, which is a sensitive tool for investigation of the iron valence and spin states as well as the type and arrangement of iron ligands (Lang *et al.,* 1966; Burda *et al.,* 1995), was applied to follow the states of hemoglobin (Hb) in the untreated and irradiated erythrocytes.

MATERIALS AND METHODS

Erythrocytes were isolated from blood *via* centrifugation in phosphate buffer, pH 7.4 using a standard method (Burda *et al.,* 2002). Five millilitres of blood was taken and heparin was added as an anti-coagulant. The blood was centrifuged (4500 rpm, 4°C) for 20 min and the pellet collected. Pelleted cells were suspended in phosphate buffer pH 7.4 (NaH_2PO_4/Na_2HPO_4, 200 mM) and centrifuged again (4500 rpm, 4°C). This procedure was repeated 3 times. Washed RBCs were suspended in the phosphate buffer at 4×10^9 red blood cells/ml. For the Mössbauer experiments about 20-fold concentrated samples having a total volume of about 1.5 ml were used. These samples were kept frozen at –80°C.

^{137}Cs was used as the source of γ rays. Its activity was 0.181 GBq and the dose rate was 0.04 mGy/min. Samples were exposed to the γ-radiation for different periods from 1 min to 45 min. TLD–LiF: Mg, Cu, P (MCP-N) detectors, with a 4.5 mm diameter and 0.9 mm thickness were used to control the doses. The detectors were calibrated before each experiment.

Hemolysis was monitored by measuring of absorption spectra of supernatant, obtained from non-irradiated and irradietd samples centrifigated after their incubation at room temperature in darkness, between 450 nm to 700 nm. A UV-2101 Shimadzu PC scanning spectrophotometer was used. Percentage of hemolysis was determined from the ratio of the area under the absorption peak centered at 577 nm of an irradiated sample to that of a corresponding non-irradiated, totally hemolyzed sample. The spontaneous hemolysis of a control (non-irradiated) sample was subtracted. All results were normalized to the amount of erythrocytes in the samples. Only freshly prepared RBCs were used in those studies.

By applying Mössbauer spectroscopy we could monitor the valence and spin state of the heme-iron in hemoglobin in the control and irradiated RBCs. This gave us information on the state of hemoglobin and its ability to bind O_2. ^{57}Co(Rh) (50 mCi) was the source of the 14.4 keV γ-radiation. The measurements were performed at 85 K. Temperature was stabilized within 0.1 K. Experimental data were fitted using Recoil Mössbauer Spectral Analysis Software (Rancourt *et al.,* 1991).

The experiments were carried out in agreement with a permission from the Bioethical Commission KBET/11/B/2009.

RESULTS

We studied the influence of low doses of γ-radiation on the stability of RBCs for four different cases of healthy and diabetic donors.

Hemolysis

The rate of spontaneous hemolysis of control, non-irradiated RBC samples, kept at room temperature, (RT = 22 ± 2°C) for 24 h, did not exceed 0.2–0.4%. This value was always subtracted from the rate of hemolysis, detected for irradiated RBCs from healthy and diabetic

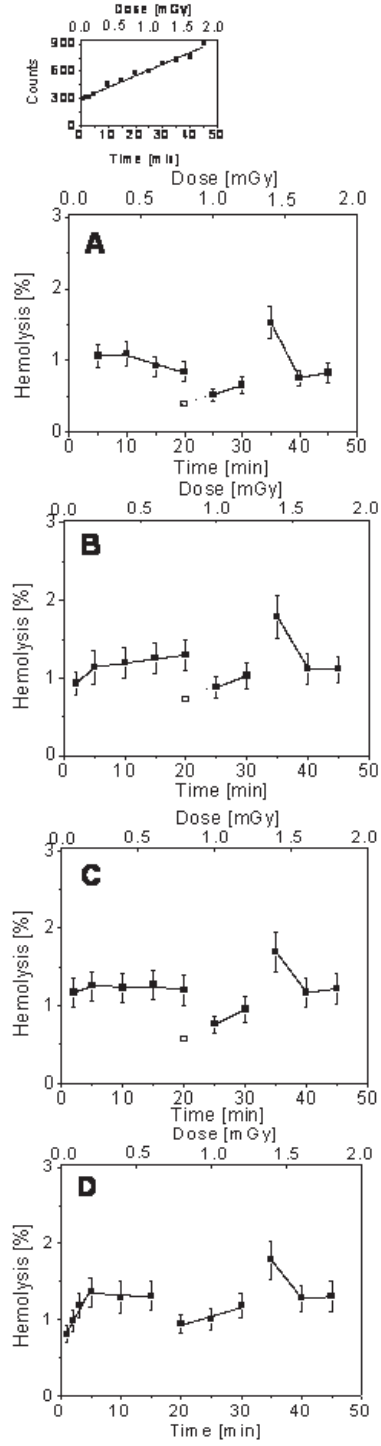

Figure 1. Dependence of hemolysis of RBCs from healthy donors on the time of irradiation by γ-rays.
Upper graph shows a calibration curve of LTD detectors. Empty symbols show extrapolated points. Each experimental point is an average from three independent measurements. Parts A–D correspond to RBCs obtained from four donors.

donors stored at RT for 24 h, which did not exceed 2% and 4%, respectively. These values are 2.5–5 times higher than that admissible by clinical guidelines for the storage of RBCs to be used in transfusion (hemolysis should be less than 0.8%). However, the solutions that support the metabolic demands of RBCs stored for transfusion contain additionally citrate, and dextrose (CPD) as well as osmoprotectants that stabilize the RBC membrane and maintain 2,3-diphosphoglycenate and ATP within the erythrocyte, for example mannitol. They are absent in our buffer. When RBCs are supplemented with additional preservative solutions (i.e., Adsol, Nutricell, or Optisol) they can be stored at 0–6°C for up to 42 days and their hemolysis does not exceed 1%. Moreover, addition of glycerol allows RBCs to be frozen at temperatures below –65°C and thus extends the period of safe storage up to 10 years (Fasano & Luban, 2008*)*. When needed, RBCs are deglycerolized by washing and such RBC units suspended in standard saline have to be transfused within 24 hours of preparation. It is known that the washing itself causes electrolyte leakage and therefore the RBC unit should be transfused as soon as possible after being washed (Weiskopf *et al.,* 2005). Thus, the rate of hemolysis detected by us in the case of non-irradiated RBCs from healthy donors is in accordance with the observations described above, although our buffer does not contain all the stabilizers and nutrients. The increased hemolysis in irradiated samples could also be expected because in our case RBCs were stored at RT only in phosphate buffer. It is known that γ-irradiation of cellular blood components at 20–50 Gy in order to prtect patients of TA-GVHD causes increased leakage of potassium and Hb even at 0–6°C and despite the presence of all the necessary components stabilizing the erythrocyte membrane (Pelsynski *et al*., 1994; Davey *et al*., 1992).

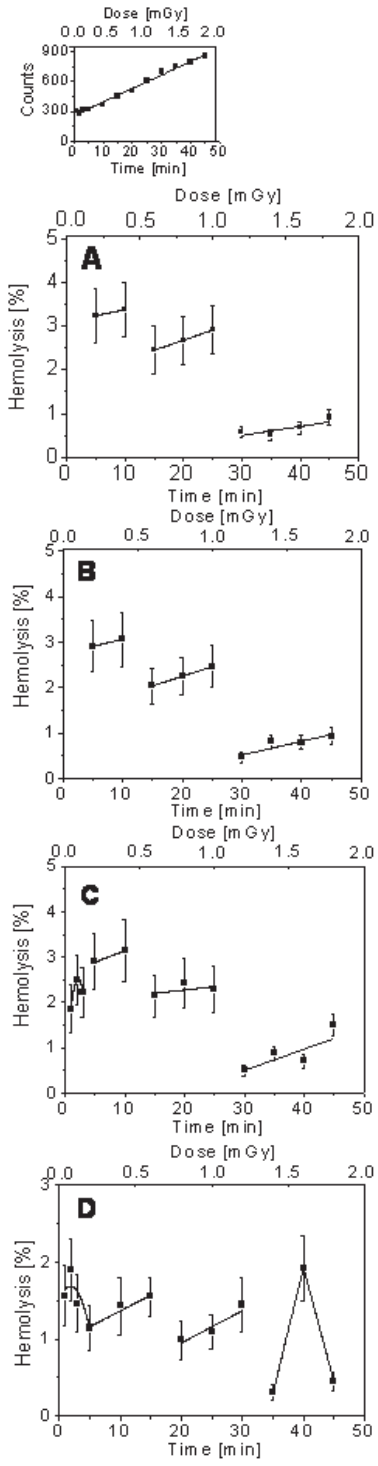

Figure 2. Dependence of hemolysis of RBCs from diabetic donors on the time of irradiation by γ-rays.
Upper graph shows a calibration curve of LTD detectors. Each experimental point is an average from three independent measurements. Parts A-D correspond to RBCs obtained from four donors.

The percentage of hemolysis of RBCs isolated from healthy and diabetic donors as a function of the γ- irradiation dose (time), are shown in Fig. 1 and Fig. 2, respectively.

In the case of RBCs isolated from healthy donors, one can distinguish five characteristic periods of irradiation when hemolysis changes monotonically (Fig. 1). The first time range from 1 to 5 minutes is characterized by an increase of hemolysis, which is then stabilized at about $1.15 \pm 0.15\%$ for the time of irradiation from 5 min to 20 min (in one case to 15 min, Fig. 1D). For 20 min of irradiation, i.e., for the dose of 0.8 mGy, a discontinuity of hemolysis is observed when its value decreases about 2-fold. Then hemolysis increases monotonically for the exposure time up to 30 min. Between 30–35 min one observes a second discontinuity of the hemolysis which increases by a factor of about 1.7. For the irradiation time $\geq$ 35 min hemolysis decreases and then is stabilized between 40–45 min on the level of about 1%.

For RBCs of diabetic donors suffering from non-insulin-dependent diabetes mellitus (diabetes mellitus of type 2) the dependence of hemolysis on the exposure time to γ-rays is different from that of RBCs from healthy donors (Fig. 2). Moreover, it depends on the degree of the severity of the disease. Figure 2A–C shows data for diabetes which cannot be managed by dietary modification alone. For these three severe diabetic cases three characteristic time periods of a monotonic increase of hemolysis are visible. They are separated by two discontinuities: (i) at 10–15 min, when hemolysis decreases to about 2 % and 1% for the more and less severe diabetes, respectively, and (ii) at 25–30 min, when hemolysis decreases by a factor of about 5. In the time range from 5 to 10 minutes hemolysis exceeds 3%, from 15 to 25 min it changes between 2–2.5%, whereas in the time range from 30 to 45 min hemolysis increases only from 0.5 to 1%. Measurements performed for irradiation times shorter than 5 min show that there is a maximum of hemolysis at 2 min of exposure to γ-rays as it was observed for the less severe diabetic case when dietary modification is sufficient (Fig. 2C and 2D). In the latter

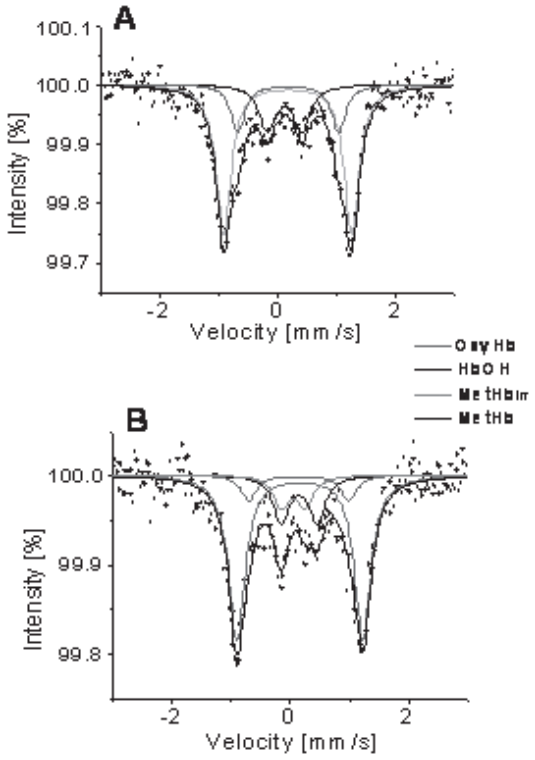

Figure 3. Mössbauer spectra of RBCs from a diabtetic donor non-irradiated (A) or irradiated (B) with 1.8 mGy of γ-rays.

case the monotonic behaviour of hemolysis resembles the data obtained for other diabetics but there are some important differences: (i) the percentage of hemolysis for the characteristic time periods of γ-irradiation between 5–25 min is lower on average by a factor of 2, (ii) the characteristic discontinuities are shifted towards shorter times of irradiation (lower doses) and are now observed at 15 min and 30 min, and (iii) an additional maximum of hemolysis is observed at 40 min.

Comparing the dependence of hemolysis on the time of irradiation, i.e., on the dose absorbed, between normal and diabetic samples, one sees that the hemolysis of RBCs from diabetic donors is about 2–3 times higher than that of healthy RBCs for doses ≤ 1.2 mGy, but is lower by a factor of 2 for doses from 1.2 to 1.8 mGy. The monotonic change of hemolysis observed in healthy cases for the dose of about 1.6 mGy disappears and an additional maximum occurs at 2 min of irradiation, i.e., for 0.08 mGy, in the diabetic cases. Moreover, the other characteristic discontinuities of hemolysis are shifted towards lower doses by 0.2 mGy (5 min shorter exposure times for the dose rate of 0.04 mGy/min). The characteritic time periods (dose ranges) seem to be related to the protective mechanisms activated within the red cells. Because it is well documented in the literature that all acute antioxidant processes related to the action of glutathione, peroxidase, SOD and catalase are diminished in the diabetic erythrocytes and result in an increased peroxidation of lipids in their membrane (Bayens & Thorpe, 1994; Uzel *et al.*, 1987; Stevens *et al.*, 2000; Thomalley *et al.*, 1996; Yoshida *et al.*, 1995; Murakami *et al.*, 1995), we infer that such acute mechanisms activated in healthy RBC membrane are attenuated in the case of diabetes. Moreover, the degree of activity of these processes depends on the dose of irradiation and time after exposure (Benderitter *et al.*, 2003). Therefore the observed differences in the membrane stability of the γ-irradiated erythrocytes from normal and diabetic individuals could be due to the different enzymatic repair processes which are activated in these cases. These mechanisms clearly show variable sensitivity, which results in the observed different discontinuities and monotonicities in the two cases studied. The results obtained for the diabetic donors confirm that the stage of the disease can be monitored by changes of the stability of RBC exposed to low doses of γ-radiation.

Mössbauer measurements

In order to check the possible influence of γ rays on the homeostasis within erythrocytes, the states of haemoglobin (Hb) inside RBC were controlled via measurements of spin and valence states of the heme-iron applying Mössbauer spectroscopy. This method is also sensitive to changes of the type and arrangement of iron ligands (G.Lang *et al.* 1966; Burda *et al.,* 1995).

We observed an effect of γ-radiation on the Hb states only for the highest applied dose of 1.8 mGy (45 min of irradiation). Representative Mössbauer spectra of non-irradiated and irradiated RBCs isolated from blood of a severe diabetic donor are shown in Fig. 3A and Fig. 3B, respectively. Because the irradiation affects the Hb states in the healthy RBCs in a similar way we do not present the data here. Values of hyperfine parameters fitted to the experimental spectra obtained for both cases are collected in Table 1.

The Mössbauer spectra of untreated RBCs can be decomposed into three components characterized by different hyperfine parameters. These doublets have comparable isomer shifts (IS) within the experimental error but they differ in the values of quadrupole splitting (QS) (Table 1). The component with the largest QS of about 2.17 mm/s characterizes a low spin ferrous state in oxygenated haemoglobin (HbO_2 = OxyHb). The doublet with QS about 1.70 mm/s has to be assigned to deoxyhemoglobin in which OH is the 6th ligand of the heme-iron (HbOH) and in this case the heme-iron is in a mixed valence state (Fe^{2+}/Fe^{3+}) having mixed spin states. The third component with QS about 0.55 mm/s comes from methemoglobin with a five-coordinated heme-iron in a high spin ferric state (MetHb) (Lang *et al.* 1966; Suzdalev, 1988). These three subspectra are detected in the Mössbauer spectra of RBCs from healthy and diabetic donors but the contributions of the components are different in these two types of RBCs. In both cases the highest contribution is that of OxyHb, however, in the diabetic RBCs its content is significantly lower by about 6%. The contribution of HbOH is comparable in healthy and diabetic RBCs but the content of MetHb is almost 2-fold higher in the latter case.

Irradiation of RBCs does not change the content of OxyHb but results in a new component in the Mössbauer spectra. The contribution of this component in healthy and diabetic RBCs is at the level of about 12% (Fig. 3B and Table 1). At the same time the contributions of HbOH and MetHb decrease. Because this new doublet is characterized by IS of about 0.07 mm/s and by QS of about 0.47 mm/s and it originates from methemoglobins it can be assigned to methemoglobin ($MetHb_{irr}$) with a five-coordinated heme-iron in a high-spin oxidized state and with the protein structure modified in proximity of the iron binding site (Mössbauer spectroscopy can detect only rearrangement of the first coordination sphere of the probed atom).

Table 1. Representative hyperfine parameters fitted to Mössbauer spectra obtained for non-irradiated and irradiated RBCs isolated from healthy and diabetic donors.
Changes of Hb states (inside RBCs) were observed only for the highest applied dose of γ-radiation, 1.8 mGy. IS, isomer shift; QS, quadrupole splitting. The line width was 0.20 ± 0.02 mm/s.

	OxyHb	HbOH	MetHb	$MetHb_{irr}$
Healthy donor, control				
CS [mm/s]	0.16 ± 0.01	0.18 ± 0.02	0.14 ± 0.05	
QS [mm/s]	2.17 ± 0.03	1.74 ± 0.08	0.55 ± 0.08	
Contribution [%]	67.4 ± 0.5	20.2 ± 1.5	12.3 ± 1.5	
Healthy donor, after irradiation				
CS [mm/s]	0.17 ± 0.01	0.15 ± 0.04	0.20 ± 0.08	0.09 ± 0.10
QS [mm/s]	2.17 ± 0.03	1.66 ± 0.11	0.74 ± 0.16	0.54 ± 0.20
Contribution [%]	65.0 ± 0.5	15.6 ± 1.5	7.0 ± 2.5	12.6 ± 2.5
Diabetic donor, control				
CS [mm/s]	0.16 ± 0.01	0.18 ± 0.04	0.11 ± 0.03	
QS [mm/s]	2.18 ± 0.04	1.71 ± 0.11	0.58 ± 0.06	
Contribution [%]	61.3 ± 0.5	18.5 ± 1.5	20.2 ± 1.5	
Diabetic donor, after irradiation				
CS [mm/s]	0.16 ± 0.01	0.14 ± 0.08	0.15 ± 0.05	0.05 ± 0.05
QS [mm/s]	2.14 ± 0.05	1.70 ± 0.26	0.61 ± 0.20	0.40 ± 0.20
Contribution [%]	62.3 ± 0.5	9.4 ± 2.5	17.1 ± 2.5	11.2 ± 2.5

DISCUSSION

RBCs are extensively used in studies of aging processes related to the ROS action because their membrane is rich in polyunsaturated lipids and hemoglobin is a strong catalyst of free radical reactions which may initiate lipid peroxidation. Therefore the RBC, being a unique carrier of oxygen, is highly susceptible to oxidative stress. It is known that peroxidation of lipids and membrane proteins alters membrane fluidity, ion transport and defensive enzymatic activities in the cell (Chiu *et al.,* 1989; Sangeetha *et al.,* 2005) and thus membrane oxidation may influence the intrinsic properties of RBC, in particular the Hb state. For example, it has been shown that the oxidative stress leads to the formation of stable complexes between Hb molecules and proteins of the RBC membrane skeleton (Snyder *et al.*, 1985). Moreover, during aging a significant decrease of the activities of many enzymes is observed in RBCs, as for example SOD, CAT or GPx (Sangeetha *et al.,* 2005). A reduced glutathione level is accompanied by a decrease of –SH groups of proteins, which suggests a direct protective role of glutathione (Asgary *et al.*, 2005). As glutathione is a reducing agent used by GPx, which protects cells from oxidative stress, depletion of glutathione caused a proportional decrease of GPx activity (Spector *et al.*, 1993). SOD and CAT participating in $O_2^{\bullet-}$ scavenging and H_2O_2 decomposition into water and oxygen, respectively, are also important for maintaining the integrity of membrane structures (Snyder *et al.,* 1985; Köteles, 1982).

We chose healthy and diabetic RBCs as subjects of our studies because it is known that the activities of important antioxidant enzymes are altered in diabetic cells than the unmodified one. In particular, it has been shown that glycated Cu,Zn-SOD has a lower enzymatic activity and its content is significantly higher in diabetic RBCs than in normal controls (Kawamura *et al.,* 1992; Arai *et al.*, 1987; Kotake *et al.,* 1998). On the other hand, a decreased activity of Cu,Zn-SOD accompanies an increased activity of CAT in diabetic RBCs (Blaszczak *et al.,* 2005). Moreover, it was shown that in diabetes there is an increase in glycooxidation and lipooxidation products and in products of reaction of proteins with dicarbonyl compounds formed by non-oxidative mechanisms and therefore in this case one should talk about carbonyl stress. A function of glutathione was recognized as the crucial protective mechanism (Baynes *et al.,* 1999) and it was found that there is a decrease in erythrocyte glutathione concentrations in clinical human diabetic cases (Thornalley *et al.*, 1996). It is well proven that the mechanisms of radiation-induced biomolecular damage involve the generation of free radicals followed by their attack on proteins, lipids and carbohydrates (Hannig *et al.*, 2000). Gamma irradiation can cause a variety of membrane changes which occur also during the aging processes, described above, for example lipid peroxidation, hydrolysis of phospholipid head groups, lipid-lipid or lipid-protein crosslinking, disulfide bridge formation, and amino acid residue damage in membrane proteins (Szweda-Lewandowska *et al.*, 1989; Lee *et al.*, 1994; Petkau, 1971; Graeub, 1994; Szweda-Lewandowska *et al.*, 2003; Komorowska *et al.*, 2007; Selim *et al.*, 2009). Therefore due to the alteration of many enzymatic pathways in diabetic RBCs resulting in a strong modification of the lipid and protein membrane components one could predict that the ionizing γ-radiation applied in our studies would influence the stability of the healthy and diabetic cells in a different way. Indeed, we observed different susceptibilities of the diabetic and healthy RBCs to the action of γ-rays at the low doses between 0.04 mGy and 1.8 mGy. Moreover, the degree of sensitivity of the RBC membranes allows us even to distinguish the stage of development of the disease. Our results show that diabetic RBCs are more sensitive to the action of γ-radiation at doses from 0.04 mGy to 1.2 mGy exhibiting not only a higher degree of hemolysis but also a shift of observed characteristic transitions towards lower doses by about 0.2 mGy and changes in the hemolysis behaviour within the characteristic irradiation time periods. The resistivity of diabetic RBCs is higher than that of healthy cells only for the doses ≥ 1.2 mGy. The Mössbauer measurements confirm that low doses of γ-radiation cannot alter the Hb states inside the RBC except at the highest applied dose of 1.8 mGy which caused formation of modified $MetHb_{irr}$ at about 12% of total Hb independently of

the RBC type. Usually, much higher doses of γ-radiation were used to observe modifications of Hb (Szweda-Lewandowska *et al*., 1989; Oshtrakh *et al.,* 1991). In addition, our experiments show that the Hb pool in diabetic RBCs contains a lower proportion of OxyHb but a higher one of MetHb. This is consistent with the observation that the percentage of glycated Hb, which is unable to bind O_2, increases in diabetic RBCs (Adak *et al.,* 2008).

The distinct discontinuities and monotonic changes of hemolysis detected by us in the healthy and diabetic RBCs suggest that various enzymatic and chemical processes are activated inside the membranes under the action of low doses of γ radiation. Their effects are stabilized within minutes. These mechanisms can be important for understanding the so called "by-stander" effects observed in new radiological approaches, in which two different doses of radiation separated in time are applied (Jayashree *et al*., 2001).

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