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## Research Paper

Effect of high glucose concentrations on human erythrocytes *in vitro*Jana Viskupicova<sup>a</sup>, Dusan Blaskovic<sup>a</sup>, Sabina Galiniak<sup>b</sup>, Mirosław Soszyński<sup>c</sup>,  
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## ABSTRACT

Exposure to high glucose concentrations *in vitro* is often employed as a model for understanding erythrocyte modifications in diabetes. However, effects of such experiments may be affected by glucose consumption during prolonged incubation and changes of cellular parameters conditioned by impaired energy balance. The aim of this study was to compare alterations in various red cell parameters in this type of experiment to differentiate between those affected by glycooxidation and those affected by energy imbalance. Erythrocytes were incubated with 5, 45 or 100 mM glucose for up to 72 h. High glucose concentrations intensified lipid peroxidation and loss of activities of erythrocyte enzymes (glutathione S-transferase and glutathione reductase). On the other hand, hemolysis, eryptosis, calcium accumulation, loss of glutathione and increase in the GSSG/GSH ratio were attenuated by high glucose apparently due to maintenance of energy supply to the cells. Loss of plasma membrane Ca<sup>2+</sup>-ATPase activity and decrease in superoxide production were not affected by glucose concentration, being seemingly determined by processes independent of both glycooxidation and energy depletion. These results point to the necessity of careful interpretation of data obtained in experiments, in which erythrocytes are subject to treatment with high glucose concentrations *in vitro*.

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## Introduction

Hyperglycemia is the most important factor in the onset and progress of diabetic complications. Diabetes mellitus is a metabolic disorder resulting in decreased number of pump units on the erythrocyte membrane, altered lipid–protein interactions, enzyme glycation and peroxidation which account for many complications [1]. Human erythrocytes are continuously exposed to glucose in plasma during their circulatory life span of 120 days. Passive transport through insulin-independent glucose transporter, GLUT1, ensures that the glucose concentration in the erythrocyte cytosol is close to that in the plasma, normally about 5 mM, and increased under conditions of hyperglycemia [2]. The erythrocytes and their membranes have always been objects of studies as they

play an important role in various physiological and metabolic events. Human erythrocyte properties such as deformability and elasticity are affected by Ca<sup>2+</sup> ions [3,4] whose level is regulated by plasma membrane Ca<sup>2+</sup>-ATPase (PMCA). A rise in internal Ca<sup>2+</sup> concentration leads to changes in cell shape and volume, increased cellular rigidity, and hemolysis [5,6].

Ca<sup>2+</sup>-ATPase activity of erythrocyte membranes is known to be decreased in diabetic patients with elevated blood glucose [7,8] and in red blood cells (RBCs) treated *ex vivo* with glucose [9], and there is evidence to suggest that such effects are related to glycation of plasma membrane Ca<sup>2+</sup>-ATPase (PMCA) [10,11]. However, Raftos et al. found that V<sub>max</sub> of active Ca<sup>2+</sup> extrusion from intact RBCs is unaffected by their exposure *in vivo* or *in vitro* to high concentration of glucose for up to 6 h [12].

The red blood cell membrane contains approximately equal weight amounts of lipids and proteins [1]. Lipid peroxidation alters the microenvironment of membrane bound enzymes by changing phospholipids and fatty acid composition [13]. In addition, membrane proteins are also glycated, which may lead to decrease in their activities [14]. Hyperglycemia in diabetes mellitus causes glycation of membrane enzymes along with oxidative stress leading to decrease in activity of Na<sup>+</sup>/K<sup>+</sup>-ATPase and other changes in erythrocyte membranes [1].

**Abbreviations:** ATPase, adenosinetriphosphatase; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; FITC, fluorescein isothiocyanate; GLUT1, glucose transporter 1; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; Hb, hemoglobin; HMF, 5-(hydroxymethyl)-2-furaldehyde; PBS, phosphate-buffered saline; PMCA, plasma membrane Ca<sup>2+</sup>-ATPase; PS, phosphatidylserine; RBCs, red blood cells; RQB, redox quenching buffer; TCA, trichloroacetic acid

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Asymmetry of transmembrane phospholipids is the characteristic feature of biological membranes. Phospholipids containing amino groups such as phosphatidylserine (PS) are localized mainly or exclusively in the inner leaflet of the plasma membrane [15]. Loss of membrane lipid asymmetry is associated with a number of physiological and pathological events [16]. Under hyperglycemic conditions, the concentration of glucose metabolite methylglyoxal is dramatically elevated in plasma as well as in erythrocytes. Increased concentration of methylglyoxal induces exposure of PS on the outer surface of erythrocytes, disrupting the asymmetry of membrane phospholipids [17]. As erythrocytes lack nucleus and mitochondria, death of erythrocytes has been called eryptosis [18] to distinguish it from apoptosis of nucleated cells. Increase of both, eryptosis and advanced glycation products, was reported in high glucose (40 and 100 mM) treated erythrocytes [19].

Incubation of erythrocytes under conditions of high glucose concentrations is usually used as a model to mimic the *in vivo* situation of hyperglycemia in diabetes. However, another aspect of this type of experiments should be taken into account: if no glucose is added during the incubation, cells incubated with initial physiological concentration of glucose utilize it during the incubation while those incubated with high glucose have longer supply of energy source. As a result, some their parameters, not so strongly affected by glycation, change less than in control erythrocytes incubated with initially physiological levels of glucose. We noted such a phenomenon in preliminary experiments; this study was aimed at comparison of behavior of several parameters critical for the function of erythrocytes during incubation with initially physiological glucose concentration (5 mM) and with high glucose (45 and 100 mM). We expected to identify by this approach those parameters whose changes are mainly dependent on glycation and those whose changes depend critically on maintenance of the energy balance of erythrocytes.

## Material and methods

### Ethical approval

The study was approved by the Research Bioethics Committee of the University of Łódź (Poland).

### Chemicals and equipment

All basic reagents were from Sigma-Aldrich Company (Poznan, Poland). All reagents used were of analytical reagent grade. All the spectrophotometric analyses were done using a Varian Cary 50 UV-vis spectrophotometer (Varian Inc., Cary, NC, USA). Fluorescence was read using an Infinite 200 PRO multimode reader (Tecan Group Ltd., Männedorf, Switzerland).

### Glucose incubation routine

Freshly drawn human whole blood was obtained from the Blood Bank in Łódź. Blood was centrifuged (2000g, 10 min, 4 °C), plasma and leukocyte buffy coat were aspirated and the erythrocyte pellet was washed three times with 4 volumes of phosphate buffered saline (PBS; 1 tablet of PBS/100 ml H<sub>2</sub>O) per 1 volume of a suspension. Residual leukocytes were removed by passing erythrocyte suspensions through a 1:1 (w/w) mixture of a cellulose and microcrystalline cellulose [20].

Washed red blood cells (RBCs) were suspended to a final hematocrit of 10% in PBS containing varying concentrations of glucose (5, 45 or 100 mM) and 10 μM ampicillin, and incubated at 37 °C for 24, 48 and 72 h with continuous mixing. The moderately high (45 mM) and very high (100 mM) glucose concentrations

employed had been applied in earlier studies by other authors [9,12,21–23]. Erythrocytes suspended in PBS-glucose (5 mM) were used as a control, because of normal blood glucose level in non-diabetic humans before meal is about 4–5.9 mM.

### Preparation of erythrocyte ghosts

Erythrocyte ghosts were prepared from washed erythrocytes according to the method of Dodge et al. with some modifications [24]. Briefly, after incubation, erythrocytes were hemolyzed on ice with 20 volumes of 20 mM phosphate buffer, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) as proteolytic inhibitors, and centrifuged at 4 °C at 20,000g for 20 min. The ghosts were resuspended in ice-cold 10 mM and then 5 mM phosphate buffer, pH 7.4 containing 0.1 mM EDTA, centrifuged and this process was continued until the ghosts were free from residual hemoglobin. Finally, the erythrocyte ghosts were resuspended in 20 mM phosphate buffer, pH 7.4. The protein concentration was estimated by the method of Lowry et al. [25].

### Flow cytometry analysis

For evaluation of eryptosis, erythrocytes (6% hematocrit) were washed in annexin-V-binding buffer containing: 125 mM NaCl, 10 mM HEPES (pH 7.4) and 5 mM CaCl<sub>2</sub>. Erythrocytes were then stained with FITC-Annexin V at a 1:20 dilution and mixed gently on a vortex mixer. After 20 min incubation in the dark at room temperature, samples were diluted 1:5, thoroughly mixed to obtain single cell suspensions, and analyzed by flow cytometry in a Becton Dickinson LSR II cytometer. Cell volume differences were estimated by forward scatter (FSC), and FITC-annexin-fluorescence intensity was measured in the FL-1 channel with an excitation wavelength of 488 nm and an emission wavelength of 530 nm as previously described [17].

Measurement of intracellular Ca<sup>2+</sup> in erythrocytes was performed according to Nicolay et al. [17]. Briefly, erythrocytes were loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) by addition of 10 μl of a Fluo-3/AM stock solution (2.0 mM in dimethylsulfoxide) to 10 ml erythrocyte suspension (0.16% hematocrit) in Ringer solution. The cells were incubated for 15 min under vigorous shaking. An additional 10 μl of a Fluo-3/AM was added and the cells were incubated 25 min. Fluo-3/AM-loaded erythrocytes were centrifuged at 1000g at 22 °C and washed twice with Ringer solution containing 0.5% bovine serum albumin (BSA) and once with albumin-free Ringer buffer. Fluo-3/AM-loaded erythrocytes were analyzed by forward and side scatter and Ca<sup>2+</sup>-dependent fluorescence intensity was measured in the FL-1 channel with an excitation wavelength of 488 nm and an emission wavelength of 530 nm using Becton Dickinson LSR II cytometer (BD Biosciences Franklin Lakes, New Jersey, USA).

### Ca<sup>2+</sup>-ATPase activity

After the treatment, ATPase activity was estimated as described previously [26]. Briefly, the membranes obtained by lysis and washing with 20 mM borate buffer instead of phosphate to avoid high phosphate background were diluted with the assay medium (final concentrations: 10 mM MgCl<sub>2</sub>, 1 mM ATP, 1 mM ethylene glycol tetraacetic acid (EGTA), 0.1 mM ouabain in 100 mM Tris-HCl buffer) and incubated at 37 °C in the presence or absence of 1 mM calcium lactate, at a protein concentration of 1.5 mg/ml, for 30 min. Samples incubated on ice served as blanks. Incubation was stopped by addition of an equal volume (70 μl) of 10% trichloroacetic acid, the samples were centrifuged and 100 μl aliquots of the supernatants were added to eppendorf tubes containing 1 ml of deionized water. Then

the tubes were added with 200  $\mu$ l of the molybdate reagent (1.75% w/v  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot \text{H}_2\text{O}$  in 3.15 M  $\text{H}_2\text{SO}_4$ ), incubated for 10 min, added with 200  $\mu$ l of 0.035% Malachite Green in 0.35% w/v polyvinyl alcohol and incubated at room temperature for another 30 min. Then absorbance of the samples was measured at 620 nm. The  $\text{Ca}^{2+}$ -AT-Pase activity was calculated as the difference between the activity measured with calcium lactate and the basal activity measured without calcium.

#### Measurement of glycosylated hemoglobin

Glycosylated hemoglobin content was measured spectrophotometrically by the method of Standefer and Eaton [27] and expressed as a percent of total hemoglobin. Glycosylation of hemoglobin was expressed as  $\mu$ moles of 5-(hydroxymethyl)-2-fur-aldehyde (HMF)/g hemoglobin.

#### Measurement of intracellular superoxide

Intracellular superoxide was estimated by labeling cells with dihydroethidine (Karlsruhe, Germany) in the dark at 37 °C for 20 min. The probe was added to a final concentration of 5  $\mu$ M. A total of  $10^5$  cells were counted with an LSRII multicolor flow cytometer (Becton Dickinson).

#### Lipid peroxidation assay

This assay is dependent upon the sensitivity of the fluorophore BODIPY (581/591)  $\text{C}_{11}$  to oxidation by peroxy/alkoxy radicals [28]. This probe readily incorporates into biological membranes and responds to free radical attack with a spectral emission shift from red to green, which can be readily monitored and quantified by flow cytometry [29].

#### Enzymatic activities

##### Catalase

Catalase activity was determined according to Aebi [30]. To 1 ml of 54 mM  $\text{H}_2\text{O}_2$  in 50 mM phosphate buffer, pH 7.0 and 1.95 ml of 50 mM phosphate buffer, pH 7.0, 50  $\mu$ l of hemolysate was added and the decrease in hydrogen peroxide concentration was measured spectrophotometrically at 240 nm, at 25 °C during 1 min. The kinetic curves were analyzed using the Cary-WinUV-software. One unit of enzyme activity was defined as the activity required to degrade 1  $\mu$ mol of hydrogen peroxide in 60 s. Enzyme activity was expressed as units per mg of Hb (U/mg Hb).

##### Glutathione S-transferase (GST)

GST activity was determined by the method described by Habig et al. [31]. 250  $\mu$ l of 0.02 M glutathione (GSH) in 0.1 M phosphate buffer, pH 6.5, was added to 750  $\mu$ l of 0.1 M phosphate buffer, pH 6.5. 10  $\mu$ l of 0.1 M chloro-2,4-dinitrobenzene (CDNB) in ethanol and 10  $\mu$ l of hemolysate was added to the cuvette. The increase of absorbance was measured at a wavelength of 340 nm for 3 min against the reagent blank. Enzyme activity was expressed as units per gram of Hb (U/g Hb).

##### Glutathione reductase (GR)

GR activity was determined using Glutathione Reductase Assay (RANDOX, GR2368).

##### Hemolysis assay

Following incubation, the samples were centrifuged (5000g, 10 min, 23 °C) and hemoglobin content in the supernatant was read at 540 nm (A). A value of 100% lysis was assigned to the supernatant of the sample with control hemolysate, obtained by

freezing and thawing the cell suspensions ( $A_1$ ). The percentage of hemolysis (H) in each assay tube was calculated by the equation:

$$H [\%] = A/A_1 * 100$$

#### Estimation of GSH and GSSG content

Glutathione determination was performed according to Senft et al. [32] as modified by Robaszekiewicz et al. [33]. Suspension of RBCs in PBS (hematocrit of 16%) treated with different concentrations of glucose was centrifuged (5000g, 10 min, 23 °C). The pellet was washed three times with PBS. Proteins of the erythrocyte pellet were precipitated with RQB-TCA buffer (20 mM HCl, 5 mM diethylenetriaminepentaacetic acid, 10 mM ascorbic acid, 5% trichloroacetic acid (TCA)), then centrifuged (5000g, 10 min, 23 °C) and the supernatant was taken for GSH and GSSG assay.

For GSH determination, 2  $\mu$ l of deproteinized supernatant diluted to 25  $\mu$ l with RQB-TCA were put on two wells (denoted '–' and '+') of a 96-well black plate. The sample '+' was added with 4  $\mu$ l of 7.5 mM N-ethylmaleimide in RQB-TCA, both samples added with 40  $\mu$ l of 1 M potassium phosphate buffer, pH 7.0, mixed for 1 min and incubated at room temperature for 5 min. Then 160  $\mu$ l of 0.1 M potassium phosphate buffer was added, followed by 25  $\mu$ l of 0.5% o-phthalaldehyde in methanol and the plate was shaken (1 min). After 30-min incubation (room temperature) the fluorescence was read at 355 nm/460 nm. The value obtained for the '–' sample was subtracted from that obtained for the '+' value and GSH concentration was read from a calibration curve obtained with glutathione as a standard.

For determining GSSG concentration, two paired samples, '–' and '+', each containing 25  $\mu$ l of deproteinized supernatant, were added with 4  $\mu$ l of 7.5 mM N-ethylmaleimide in RQB-TCA and 40  $\mu$ l of 1 M potassium phosphate buffer. Then, 5  $\mu$ l of 100 mM sodium dithionite in RQB-TCA was introduced into the sample '+'. The mixture was incubated at room temperature for 60 min. The remaining part of the procedure was the same as for GSH estimation. The calibration curve was prepared with GSSG.

#### Hemoglobin assay

Hemoglobin (Hb) concentration was estimated according to the Drabkin's method [34]. 20  $\mu$ l of the hemolysate was added to 5000  $\mu$ l of the Drabkin reagent (0.03%  $\text{K}_3[\text{Fe}(\text{CN})_6]$ , 0.1%  $\text{NaHCO}_3$ , 0.005% KCN) and incubated for 15 min; then the absorbance was measured at a wavelength of 540 nm against a reagent blank. All the spectrophotometric analyses were done using a Varian Cary 50 UV-vis spectrophotometer (Varian Inc., Cary, NC, USA). Fluorescence was read using an Infinite 200 PRO multimode reader (Tecan Group Ltd., Switzerland).

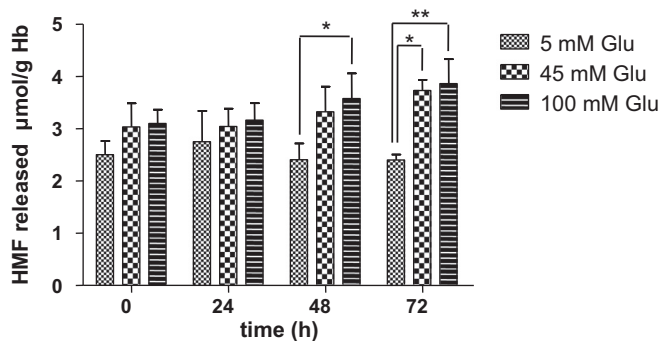
#### Statistical analysis

Data were expressed as means of the percentage of control  $\pm$  S. D. Statistical comparison between groups was performed using the one-way ANOVA followed by Bonferroni test for mutual comparison of treated samples with control and by STATISTICS, version 10 (2010; StatSoft Inc., Tulsa, OK, USA). Differences among means were considered significant at \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .

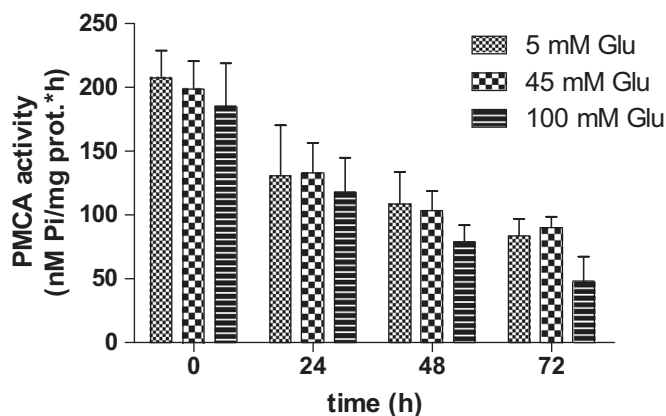
## Results

The level of glycosylated hemoglobin in control samples (5 mM glucose) was practically not changed with increasing time of incubation. High concentrations of glucose induced glycation of hemoglobin in 48 h (100 mM glucose) and 72 h of incubation (45 and 100 mM glucose) (Fig. 1).

When erythrocytes were incubated with normal (5 mM), high (45 mM) and extremely high (100 mM) concentrations of glucose



**Fig. 1.** Hemoglobin glycation after incubation of erythrocytes with high glucose. 5-Hydroxymethylfurfural (HMF) formation was used to estimate hemoglobin glycation. Incubation of RBCs with 5 mM glucose for 24, 48 and 72 h did not result to any changes in the level of glycated hemoglobin. Significant increase ( $p < 0.05$ ) of glycated hemoglobin by 100 mM glucose at 48 h of incubation and by 45 mM glucose ( $p < 0.05$ ) and 100 mM glucose ( $p < 0.01$ ) at 72 h of incubation was observed.

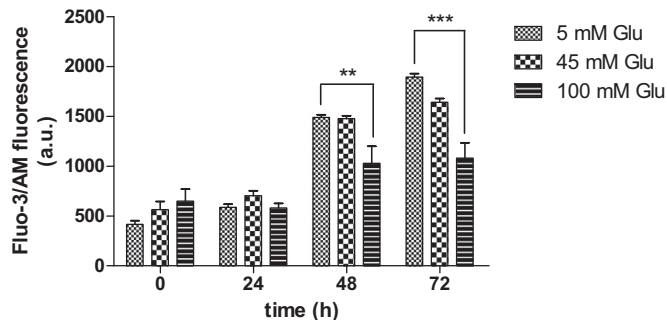


**Fig. 2.** Effect of incubation in various glucose concentrations on erythrocyte membrane PMCA activity. Significant decrease of PMCA activities of all glucose concentrations in comparison with starting (0 h) values were observed for all time intervals. No significant differences were observed by comparing high level glucose-treated cells with control cells for the same time intervals.

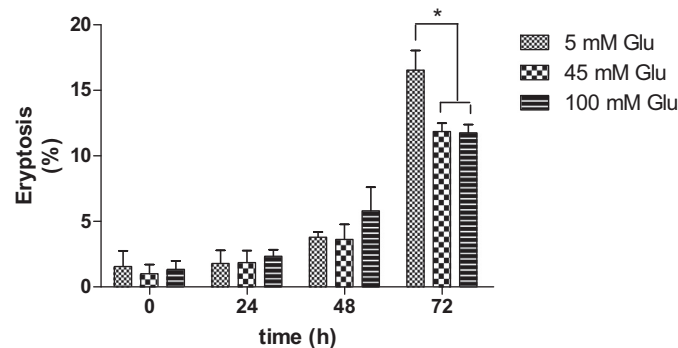
for 24, 48 and 72 h, PMCA activity decreased with increasing incubation time in control sample (5 mM glucose) as well as in samples incubated with 45 mM or 100 mM glucose. There was no significant effect of glucose concentration on the loss of PMCA activity (Fig. 2).

Cytosolic calcium concentration increased in control sample (5 mM glucose) incubated for up to 48 and 72 h. The presence of high glucose concentrations (45 mM and 100 mM) decreased the level of calcium after incubation for up to 48 h when compared to samples with 5 mM glucose (Fig. 3). Eryptosis significantly increased only after incubation of the cells with 5 mM glucose for up to 72 h. High glucose levels attenuated eryptosis in comparison with control samples (5 mM glucose) after 72-h incubation (Fig. 4). Increase of hemolysis was observed with 5 mM glucose and incubation time of up to 48 and 72 h. Under conditions of high concentrations of glucose hemolysis was attenuated and observed only after the longest incubation time (72 h; Table 1).

Effect of high glucose on erythrocyte redox balance was determined by evaluation of intracellular superoxide level, lipid peroxidation, the level of reduced and oxidized glutathione. In addition, activities of redox-related enzymes (catalase, GR and GST) were estimated as markers of possible redox imbalance (Table 1).



**Fig. 3.** Effect of incubation in various glucose concentrations on intracellular calcium level of erythrocytes. Intracellular calcium was determined with Fluo-3/AM using flow cytometry. Significant increase of intracellular calcium in comparison with starting values were observed for all glucose concentrations after incubation of erythrocytes for 48 and 72 h ( $p < 0.001$ ). Significant decrease ( $p < 0.01$ ) of calcium level by 100 mM glucose in comparison with low level of glucose at 48 h of incubation was observed. Significant decrease ( $p < 0.001$ ) of calcium level by 100 mM glucose to compare with low level of glucose at 72 h of incubation was found.



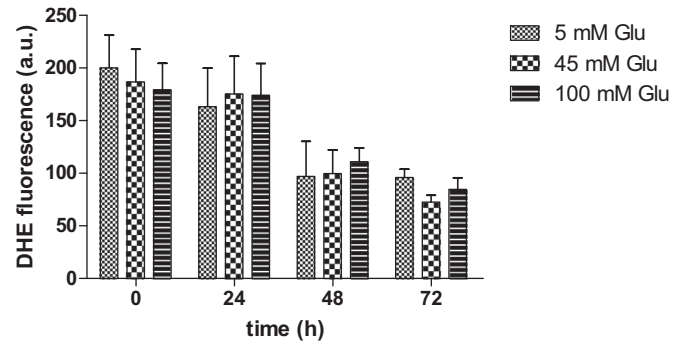
**Fig. 4.** Effect of erythrocyte incubation in various glucose concentrations on eryptosis. Eryptosis was measured by exposure of phosphatidylserine by fluorescence of Annexin-V using flow cytometry. All glucose concentrations induced significant increase of eryptosis in comparison with starting values (0 h) after incubation of erythrocytes for 72 h ( $p < 0.001$ ). Significant decrease ( $p < 0.01$ ) of eryptosis by 45 and 100 mM glucose in comparison with 5 mM glucose after 72 h was observed.

The intracellular content of superoxide declined with increasing incubation time in control erythrocytes and in cells with both high concentrations of glucose (Fig. 5). No significant differences in the superoxide level were found between samples with high glucose (45 mM and 100 mM) in comparison with the control (5 mM glucose). Lipid peroxidation had a tendency to a time-dependent increase in all samples tested, which, however, did not reach statistical significance except for samples containing 100 mM of glucose where the increase after 72 h was statistically significant (Fig. 6). The activity of catalase decreased drastically in control samples (containing 5 mM glucose) after 48 h and 72 h of incubation (Table 1). 45 mM glucose prevented the decrease of catalase activity observed in control cells; however, a decrease was observed in samples incubated with 100 mM glucose. In control samples (5 mM glucose) significant decreases of both GSH and GSSG were observed within the whole period of time tested (24–72 h). Glucose (45 and 100 mM) attenuated both GSH and GSSG decrease after 48 and 72 h. The activity of GST was decreased by prolonged incubation at high glucose concentrations (48 h for 45 mM glucose, 48 h and 72 h for 100 mM glucose). GR activity was decreased by incubation in high glucose (24–72 h for 45 mM glucose, 48 and 72 h for 100 mM glucose) (Table 1).



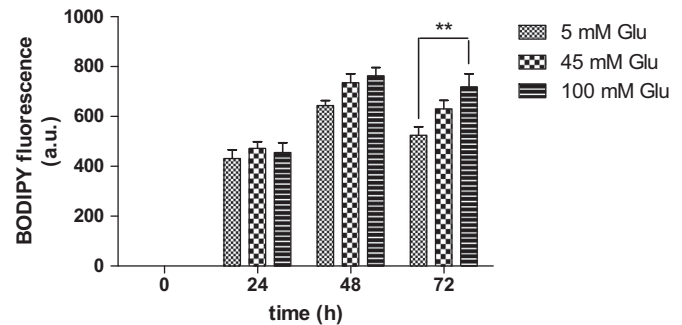
**Table 1**  
Effect of incubation in various glucose concentrations on selected erythrocyte parameters: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

Concentration of glucose (mM)	45				100			
	0				0			
	0	24	48	72	0	24	48	72
Incubation time (h)	0	24	48	72	0	24	48	72
GST (U/g Hb)	2.1 ± 0.05	1.8 ± 0.11*	2.0 ± 0.05	2.0 ± 0.06	2.0 ± 0.01	2 ± 0.08	1.8 ± 0.06**	1.9 ± 0.09
Catalase (U/mg Hb)	397.1 ± 25.39	397.1 ± 16.57	310.3 ± 2.37***	228.5 ± 8.02***	396.2 ± 32.11	417.5 ± 11.85	351.9 ± 16.46	402.2 ± 10.04
Glutathione reductase (U/g Hb)	9.2 ± 0.14	8.6 ± 0.13*	9.1 ± 0.38	8.9 ± 0.23	8.8 ± 0.17	8.2 ± 0.17**	7.6 ± 0.11**	8.3 ± 0.15***
Hemolysis (%)	7.9 ± 0.83	8.8 ± 0.43	13.6 ± 0.6***	20.9 ± 0.65***	8.1 ± 1.19	8.5 ± 2.13	10.2 ± 1.45	14 ± 0.56***
GSH (µmol/g Hb)	6.2 ± 0.18	1.6 ± 0.06***	1 ± 0.05***	0.9 ± 0.05***	6.2 ± 0.16	4.6 ± 0.14***	4.5 ± 0.12***	3.5 ± 0.11***
GSSG (µmol/g Hb)	0.5 ± 0.07	0.2 ± 0.03**	0.2 ± 0.02***	0.1 ± 0.02***	0.5 ± 0.07	0.4 ± 0.05	0.3 ± 0.04**	0.3 ± 0.03***
GSSG/GSH ratio	0.07 ± 0.01	0.14 ± 0.01***	0.15 ± 0.01***	0.17 ± 0.01***	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
	0	24	48	72	0	24	48	72
Catalase (U/mg Hb)	2.1 ± 0.1	2.3 ± 0.12	1.8 ± 0.05***	1.8 ± 0.05***	2.1 ± 0.1	2.3 ± 0.12	1.8 ± 0.05***	1.7 ± 0.05***
Glutathione reductase (U/g Hb)	427.2 ± 42.82	454.3 ± 27.04	363.2 ± 8.01	363.2 ± 8.01	427.2 ± 42.82	454.3 ± 27.04	363.2 ± 8.01	350.8 ± 1.95*
Hemolysis (%)	8.7 ± 0.38	9.2 ± 0.21	7.5 ± 0.26**	7.5 ± 0.26**	8.7 ± 0.38	9.2 ± 0.21	7.5 ± 0.26**	7.5 ± 0.39**
GSH (µmol/g Hb)	8.2 ± 1.1	8.3 ± 1.72	9.7 ± 1.1	9.7 ± 1.1	8.2 ± 1.1	8.3 ± 1.72	9.7 ± 1.1	12 ± 0.54**
GSSG (µmol/g Hb)	6.4 ± 0.16	6 ± 0.17*	5.5 ± 0.14***	5.5 ± 0.14***	6.4 ± 0.16	6 ± 0.17*	5.5 ± 0.14***	3.8 ± 0.11***
GSSG/GSH ratio	0.5 ± 0.06	0.4 ± 0.06	0.4 ± 0.05	0.4 ± 0.05	0.5 ± 0.06	0.4 ± 0.06	0.4 ± 0.05	0.3 ± 0.04*
	0	24	48	72	0	24	48	72
GST (U/g Hb)	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01



**Fig. 5.** Effect of incubation in various glucose concentrations on superoxide production in erythrocytes.

Dihydroethidine (DHE) was used to measure superoxide level. All glucose concentrations induced significant decrease of superoxide level in comparison with starting values (0 h) after incubation of erythrocytes for 48 and 72 h ( $p < 0.05$ ).



**Fig. 6.** Lipid peroxidation in erythrocytes after incubation in various glucose concentrations. All glucose concentrations induced significant increase of lipid peroxidation in comparison with starting values (0 h) after incubation of erythrocytes for 24, 48 and 72 h ( $p < 0.001$ ). Significant increase ( $p < 0.01$ ) of lipid peroxidation induced by 100 mM glucose in comparison with 5 mM glucose after 72 h of incubation was observed.

## Discussion

Glycated hemoglobin is a minor fraction of hemoglobin in human erythrocytes, generated *in vivo* by nonenzymatic binding of glucose to N-terminal amino acids of beta chains of hemoglobin A [35,36]. The extent of hemoglobin glycation depends on the age of erythrocytes and glucose concentration in blood. Amount of glycated hemoglobin is higher in older as well as in diabetic erythrocytes. Both intracellular and membrane proteins of erythrocytes are glycosylated under hyperglycemic conditions [36]. We observed time-dependent increase in hemoglobin glycation under high glucose conditions.

Energy production in the form of ATP is necessary for maintaining erythrocyte function: keeping ion gradients across the membrane via ATP-dependent membrane pumps, maintenance of asymmetry of the membrane phospholipids, synthesis of glutathione and other metabolites, and protection of hemoglobin, enzymes and membrane proteins against oxidative impairment [37].

In the course of incubation within 0, 24, 48 and 72 h of control human erythrocytes with 5 mM glucose in PBS, degradative processes were observed in this study: decrease of  $\text{Ca}^{2+}$ -ATPase activity, increase of cytosolic calcium level, increase of eryptosis, hemolysis, lipid peroxidation and decrease of antioxidant potential as indicated by increasing GSSG/GSH ratio. This may be caused mainly by consumption of glucose as a main substrate for energetic metabolism of erythrocytes [38], associated with decrease of ATP concentration. Energy in erythrocytes is generated only by glycolysis and the pentose phosphate shuttle as these cells lack

mitochondria. The presence of high levels of glucose (45 and 100 mM) did not affect the decrease in PMCA activity.

Intracellular calcium content in control samples, as well as in erythrocytes treated with high concentrations of glucose increased after 48 and 72 h, correlating with reduction of PMCA activity. Calcium content in erythrocytes treated with 45 mM glucose for up to 24, 48 and 72 h and with 100 mM glucose for up to 24 h was practically equal to that of control erythrocytes. Raftos et al. [12] reported normal (not changed) calcium extrusion in erythrocytes after incubation of intact RBCs in the presence of 30–100 mM glucose for up to 6 h. However, after incubation of erythrocytes with 100 mM glucose for up to 48 and 72 h, decrease in calcium content was observed in comparison with samples incubated with 5 mM glucose. The decrease of calcium under conditions of high glucose may be due to an adaptive mechanisms operating by alternative calcium transport out of cell via NCX transporter. Increased calcium concentration induces eryptosis in erythrocytes [18]. To assess the extent of eryptosis, we tested level the phosphatidylserine (PS) exposure, which is a marker of eryptosis. Eryptosis increased in control samples (5 mM glucose) incubated for up to 72 h and was lower in RBCs incubated with 45 and 100 mM glucose for up to 72 h than in the control. Energy depletion is well known to induce and mediate apoptosis [39]. Apparently, cells incubated with high glucose, having available source of metabolic energy, were able to counteract apoptosis to some extent.

High glucose concentration has been shown to generate superoxide and other free radicals from the autooxidation of glucose and glycated proteins with concomitant increase in cellular lipid peroxidation, membrane damage in different cell systems and in diabetic blood [40–42]. We observed decrease of superoxide level independent of glucose concentration up to 72 h. This finding is somewhat puzzling since hemoglobin autooxidation is believed to be the main source of superoxide in erythrocytes [43] and this reaction is expected to be accelerated by glutathione loss [44]. Moreover, dramatic differences in the behavior of GSH concentration between control and high glucose-treated erythrocytes were not reflected by the rate of superoxide production. Another source of superoxide in erythrocytes may be NADPH oxidase [45]. The loss of activity of this enzyme may be the main reason for the observed loss of superoxide production in erythrocytes.

In control erythrocytes a progressive loss of GSH concentration and increase in the GSSG/GSH ratio was observed. Glutathione is an important intracellular antioxidant maintained in the reduced state by the pentose phosphate cycle (which utilizes 3–11% of glucose in erythrocytes), which is a single pathway generating NADPH, necessary for reduction of GSSG [46]. The loss of GSH and the increase in the GSSG/GSH was prevented by high glucose.

Oxidative stress in erythrocytes induced by glucose was reported to increase membrane lipid peroxidation in diabetic tissue pathology [1,39,46]. Also high glucose treated human erythrocytes enhanced lipid peroxidation [47]. In our study, high glucose concentrations enhanced lipid peroxidation.

Hemolysis was attenuated by high glucose concentrations. This result contradicts the data of Marar [48] who found hemolysis to increase with increasing glucose concentrations after 24-h incubation at 37 °C. The reason for this discrepancy is unclear.

A factor not addresses in the present study is the osmotic effect induced by high glucose concentrations. The effect is changeable with time since as glucose is consumed during the incubation. However, it may contribute to the decrease in hemolysis by high glucose concentrations.

In summary, we observed three types of behavior of erythrocyte parameters studied on glucose concentration. Lipid peroxidation, loss of activities of GST and GR were intensified by high glucose concentrations. Apparently, these parameters are significantly affected by glycoxidation. Loss of PMCA activity and superoxide production

were independent of glucose concentration and most probably conditioned by processes independent of glycation and also not significantly dependent on the energy balance of erythrocytes. Hemolysis, eryptosis, calcium accumulation, loss of glutathione and increase in the GSSG/GSH ratio were attenuated by high glucose apparently due to maintenance of energy supply to the cells. Catalase activity decreased in control erythrocytes, most probably due to depletion of NADPH necessary for maintaining the enzyme activity [49] and in erythrocytes incubated with 100 mM but not 45 mM glucose, apparently due to glycation. These results point to the necessity of careful interpretation of data obtained in experiments in which erythrocytes are subject to treatment with high glucose concentrations *in vitro*.

## Conflict of interest

The authors declare no conflict of interest.

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