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Original article

EFFECT OF OLIGOSACCHARIDES ON ENZYMES OF CARBOHYDRATE METABOLISM AND ANTIOXIDANT PROTECTION IN *IN VITRO* TREATED ERYTHROCYTES UNDER CONDITIONS OF HYPERGLYCEMIA

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ABSTRACT

Introduction: The purpose of this experiment is to examine the effect of different oligosaccharides with proven prebiotic effects on enzymes of carbohydrate metabolism and the antioxidant protection of erythrocytes in vitro under conditions of hyperglycemia.

Materials and methods: This experiment included 10 healthy men (27±3 years of age). The isolated erythrocytes were treated with 1% and 5% solutions of the following oligosaccharides: lactulose, inulin, galactooligosaccharide and fructooligosaccharide in the presence of 5mM, 50mM and 100mM glucose. After incubation, for 2 hours at 37 °C, the erythrocytes were lysed, and the supernatant was used for analyses of lactate dehydrogenase, hexokinase and glutathione reductase. FRAP (Ferric reducing antioxidant power) method was used for determining the total antioxidant activity of erythrocytes.

Results: Lactate dehydrogenase was decreased in the presence of 5% lactulose in groups with 50mM and 100 mM Glc. An increase in the activity of glutathione reductase under severe hyperglycemia (100mM glucose) was observed after treatment with: 1% lactulose, 1% inulin, 1% galactooligosaccharide, 1% and 5% fructooligosaccharides ($p<0.005$). A significant difference in the enzymatic activity of hexokinase was found in all groups ($p<0.05$) and of glutathione reductase only in the control group as well as in the groups treated with 1% lactulose, 1% galactooligosaccharide, 1% and 5% fructooligosaccharides**.**

Conclusions: Galactooligosaccharides 1% and fructooligosaccharides 1% and 5% cause a statistically significant increase of the enzymatic activities of hexokinase and glutathione reductase in in vitro hyperglycemia induced by 100 mM glucose, as well as an increase in FRAP.

Keywords: prebiotics, oligosaccharides, hyperglycemia, erythrocytes, antioxidant protection,

INTRODUCTION

Prebiotics are indigestible nutrients, which selectively stimulate the growth, composition and activity of the intestinal microflora and therefore improve the health and the wellbeing of the host. They are not affected by the severe conditions in the gastrointestinal tract and cause more changes to the microflora compared to probiotics [1, 2].

In the metabolism of prebiotics by the beneficial microflora, the increased concentration of short-chain fatty acids (SCFAs) (acetate, propionate, butyrate) is well established [3, 4]. They bind specific cell receptors – G-protein coupled receptors (GPCR41 and GPCR43), which boost the normal functioning of the intestinal mucosa and stimulate the immune system by inhibiting the production of interleukin (IL) -6, IL-1β, tumor necrosis factor-α and nitric oxide, but they increase the production of anti-inflammatory cytokine IL-10 by stimulating the GPCRs inhibiting the histone deacetylase (HDAC) [5]. Prebiotics can improve glucose homeostasis through two separate mechanisms: (1) regulation of the glucagon like peptide 1 (GLP-1) for improving the function of the β-cells and (2) decreasing the permeability of the intestinal wall for bacterial lipopolysaccharides (LPS) from gram-negative microorganisms, which could lead to an improvement of the insulin sensitivity [6].

Prebiotics are mainly of carbohydrate origin and refer to the following oligosaccharides: fructans (inulin and fructooligosaccharides – FOS) [7, 8], galactans (galactooligosaccharides – GOS) [8, 9], lactulose [10], xylooligosaccharides (XOS) [11, 8, 12], manooligosaccharides (MOS) [13], oligosaccharides from human milk (HMO) [8].

Diabetes mellitus (DM) represents a group of metabolic violations characterized by the presence of hyperglycemia in the absence of treatment. The heterogeneous etiopathology includes defects in the secretion of insulin, in insulin's action or in both, as well as disorders of the metabolism of carbohydrates, lipids and proteins. In 2014 there were 422 million people registered with diabetes worldwide, and their count is expected to grow to 629 million by 2045 if the disease is not limited [14]. According

to the International Diabetes Federation (IDF), in 2019, there were approximately 463 million adults with diabetes, which proves the tendency of the cases to rise.

One of the main factors for the progression of diabetes complications is hyperglycemia. It is associated with complications on molecular, cellular and organ level, which lead to cardiovascular diseases, neuropathies, nephropathies, blindness, limb amputations, etc. Although erythrocytes are energetically dependent on glucose, hyperglycemia leads to a decrease in the number of Na^+/K^+-ATPases and Ca^{2+}-ATPases in their membranes, glycation of proteins (hemoglobin and enzymes), lipid peroxidation, a decrease of their mobility, shortening of their half-life, etc. [15,16]. Glucose enters erythrocytes through non-insulin dependent transporter GLUT1. Because of the lack of mitochondria, the metabolism of glucose, which follows the Embden-Meyerhof pathway, leads to the formation of lactate or pyruvate and the generation of adenosine triphosphate (ATP), required for various metabolic processes as well as for the maintenance of the shape of erythrocytes. 3-11 % of the glucose used by erythrocytes undergoes the pentose phosphate pathway in physiologic conditions. This pathway plays a significant role in the maintenance of the oxidizable components of erythrocytes in a reduced state by providing a redox potential in the form of reduced nicotinamide adenine dinucleotide phosphate (NADPH) [17]. Glycolysis is strictly regulated by three irreversible reactions catalyzed by: hexokinase (HK; EC 2.7.1.1), phosphofructokinase (PFK; EC 2.7.1.11) and pyruvate kinase (PK; EC 2.7.1.40). It has also been reported that the intracellular concentrations of the intermediate metabolites of glycolysis significantly change in erythrocytes of patients with diabetes and also that there is lactic acidosis due to the increased activity of lactate dehydrogenase (LDH) [18]. HK has four isoforms and the first isoform of HK (HK I) is expressed mainly in tissues and erythrocytes [19]. Erythrocytes are constantly under oxidative stress because of the elevated oxygen level, the presence of hemoglobin, fatty acids in the membranes, etc. Oxidative processes can lead to a number of disorders of erythrocyte metabolism as well as a full loss of function. Erythrocytes have several physiological defense mechanisms against intracellular oxidative stress, and these are the antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and glutathione reductase (GR). GR is important for the maintenance of the levels of reduced glutathione [20].

The purpose of the present experiment is to examine the effect of different oligosaccharides with proven prebiotic effect on enzymes of carbohydrate metabolism and the antioxidant protection of erythrocytes in vitro under conditions of hyperglycemia.

MATERIALS AND METHODS

Ethical approval

This experiment was conducted in accordance with the ethical principles of The Declaration of Helsinki on Human Experiments. All procedures were approved by the ethical commission (protocol No.2/13.06.2019) of the Medical University of Plovdiv. Written informed approval was obtained by all participants before the study.

Reagents and equipment

Reagents provided by Chema Diagnostic (Monsano An, Italy) were used for the determination of lactate dehydrogenase (LDH) activity. The hexokinase (HK) and the glutathione reductase (GR) were determined with reagents provided by Sigma Aldrich, US. The spectrophotometric measurements were performed on an 800 DU spectrophotometer (Beckman Coulter®, Brea, CA, the U.S.).

Isolation, treatment and lysis of erythrocytes

The donors included in this experiment were 10 young men, 27±3 years of age, clinically healthy, with no chronic diseases, who do not administer any drugs. Each sample was processed and analysed separately. Erythrocytes were isolated from blood, taken by venipuncture in vacuum containers containing EDTA as an anticoagulant. Initially, the blood was centrifuged at 3000 rpm for 20 minutes at 4 °C. The plasma and the lymphocytes were separated by aspiration, and the remaining cells were washed by adding 4 volumes of PBS buffer, pH 7.4. Then they were centrifuged at 3000 rpm for 10 minutes at 4°C. The process was repeated twice more (three washings all together). The erythrocytes were treated with 1% and 5% solutions of the following oligosaccharides (diluted in PBS buffer, pH 7.4): lactulose, inulin, galactooligosaccharide (GOS) and fructooligosaccharide (FOS) in the presence of 5mM, 50mM and 100mM glucose (Glc), which caused hyperglycemia. The chosen concentrations of glucose have been applied in several studies, which aimed to explain the effect of high glucose concentration on erythrocyte metabolism in *in vitro* conditions [15, 28, 29]. The samples with the presence of 5mM glucose represented a control group and simulated the normal physiological conditions in erythrocytes.

After that, the samples were placed in an incubator with a shaker for 2 hours at 37 °C. The treated erythrocytes were centrifuged for 10 minutes at 3000 rpm. The supernatant was removed, and the erythrocytes were then washed with PBS buffer.

The erythrocytes were lysed by adding ice-cold water in proportion 1:20 [21]. They were centrifuged for 10 minutes at 7000 rpm. The separated supernatant was used for the measurement of the relevant enzymatic activities.

Enzyme analyzes

Lactate dehydrogenase (LDH) is an enzyme, catalyzing a reversible reaction, which reduces pyruvate to lactate in the presence of NADH, which is converted to NAD^+ [22]. The rate of the conversion of NADH to NAD^+ measured at 340 nm is proportional to LDH activity. Reagents provided by Chema Diagnostic (Monsano An, Italy) were used for the determination of LDH activity. One ml of reagent 1 was mixed with 10 µl of erythrocytes hemolysate, the mixture was incubated for 5min at 37 °C and after that 250 µl of reagent 2 were added. A measurement of the absorbance at λ = 340 nm against a control, which contained distilled water instead of the hemolysate was performed. Other 5 readings at a 60 seconds interval were performed. Calculated ΔA/min was multiplied by the factor 20080 and U/ml was obtained. The specific enzyme activity was determined by the formula: U/mg= (U/ml)/(mg/ml protein).

The method of Bergmeyer and Walter was used for

the determination of hexokinase (HK) activity [23]. It is based on the measurement of the absorption of NADPH, accumulated in the solution due to the glucose-6-phosphate dehydrogenase reaction, at 340 nm. The reaction mixture contained: 1 ml Tris Buffer (Tris-HCl) pH 7.6, 1 ml 555 mM glucose, 100 µl 19 mM ATP, 200 µl 100 mM $MgCl_2$, 200 µl 14 mM NADP, 20 µl 125 U/ml glucose-6-phosphate dehydrogenase (Sigma-Aldrich, cat. No. G8404) and 50 µl erythrocyte hemolysate, respectively 50 µl distilled water in the blank. The contents was mixed by inversion and an increase in the absorption at 340 nm every 30 sec for 5 minutes was monitored. The equation for calculation of the enzyme activity, that was used, is: Units/mL enzyme = (ΔA340nm/min Test - ΔA340nm/min Blank)(2.57)(df)/ (6.22)(0.05)

Where:

2.57 = Total volume (in milliliters) of assay

df = Dilution factor

6.22 = Millimolar extinction coefficient of â-NADPH at 340 nm

0.05 = Volume (in milliliter) of enzyme used

The specific enzyme activity was determined by the formula: U/mg= (U/ml)/(mg/ml protein).

In order to study the antioxidant effects of the oligosaccharides, the enzymatic activity of GR was studied [24]. The oxidized glutathione (GSSG) is reduced to two molecules of reduced glutathione (GSH) in the presence of NADPH. The reaction mixture contained: 650 µl of distilled water, 1.5 ml of 100 mM potassium phosphate buffer, pH 7.6, with 3.4 mM EDTA, 100 ìl 30 mM GSSG, 350 µl 0.8 mM NADPH, 300 µl 1.0 % bovine serum albumin and 100 µl erythrocyte hemolysate. Blank contained 400 µl 1.0 % bovine serum albumin. The contents was mixed by inversion and a decrease in the absorption at 340 nm every 30 sec for 5 minutes was monitored. The equation for calculation of the enzyme activity, that was used, is: Units/mL enzyme = (ΔA340 nm/min Test - ΔA340nm/min Blank)(3)(df)/ (6.22)(0.1)

Where:

3 = Total volume (in milliliters) of assay

df = Dilution factor

6.22 = Millimolar extinction coefficient of β-NADPH at 340 nm

0.1 = Volume (in milliliter) of enzyme used

The specific enzyme activity was determined by the formula: U/mg= (U/ml)/(mg/ml protein).

Determination of the total antioxidant activity of erythrocytes

For this purpose, the FRAP (Ferric reducing antioxidant power) method of Benzie and Strain with certain changes was used [25]. FRAP reagent was made immediately before use by mixing 50ml of acetate buffer (300 mM, pH 3.6), 5 ml of the TPTZ stock solution (10 mM) and 5 ml of $FeCl_3$ (20 mM). Then 2.85 ml FRAP reagent was mixed with 0.15ml of the supernatant, the mixture was incubated for 30 minutes at 37 °C in the dark, and the absorption was measured at 593 nm. The results were expressed in micromoles Trolox equivalent.

The level of total protein needed to express the specific enzymatic activity of certain enzymes relative to a milligram of protein (U/mg protein) was measured by the method of Bradford [26].

Statistical methods

The statistical analyzes were made with the statistical program SPSS, version 19.0 (SPSS Inc., Chicago, IL, USA). The descriptive statistics include a median and 95% confidence interval (CI) because of the non-Gaussian distribution of the results. The Mann-Whitney U-test was used for the comparison of quantitative variables with a non-Gaussian distribution between two independent groups. The Kruskal-Wallis test was applied for the comparison of the quantitative variables of more than 2 independent groups.

RESULTS

A statistically significant difference in the enzymatic activity of LDH ($p<0.05$) in the three subgroups with 5mM glucose, 50mM glucose and 100mM glucose (5mM Glc, 50mM Glc, 100mM Glc) was detected in samples treated with 5% lactulose, 1% inulin, 1% GOS, 5% GOS, 1 % FOS and 5% FOS (Table 1). A significant difference in the enzymatic activity of HK was detected in all groups ($p<0.05$) (Table 1) and of GR - in the control group as well as in the groups treated with 1% lactulose, 1% GOS, 1% FOS and 5% FOS (Table 1).

Table 1. Descriptive statistics of LDH, HK and GR (U/mg protein) of erythrocytes, treated with oligosaccharides in the presence of 5mM, 50mM and 100mM glucose

		LDH			HK			GR		
Groups	**Subgroups**	**Median**	**95% CI**	**p (Kruskal-Wallis Test)**	**Median**	**95% CI**	**p (Kruskal-Wallis Test)**	**Median**	**95% CI**	**p (Kruskal-Wallis Test)**
Control (n=10)	5mM Glc	0,95	0,88-1,16	0.360	0.01	0.00-0.002	**0.001***	0.16	0.15-0.18	**0.039***
	50mM Glc	0,89	0.74-1.04		0.07	0.06-0.08		0.14	0.12-0.16	
	100mM Glc	1,01	0.87-1.14		0.08	0.07-0.09		0.18	0.15-0.20	

	5mM Glc	0,86	0.72-1.06		0.04	0.01-0.05		0.14	0.11-0.17	
Lactulose 1% (n=10)	50mM Glc	0,81	0.68-1.05	0.747	0.05	0.04-0.07	**0.022***	0.13	0.11-0.17	**0.000***
	100mM Glc	0,85	0.63-1.07		0.07	0.04-0.12		0.26	0.24-0.30	
	5mM Glc	0,94	0.82-1.14		0.10	0.08-0.11		0.17	0.13-0.18	
Lactulose 5% (n=10)	50mM Glc	0,7	0.66-0.77	**0.006***	0.09	0.07-0.10	**0.002***	0.17	0.13-0.20	0.253
	100mM Glc	0.67	0.57-0.82		0.04	0.02-0.06		0.19	0.16-0.24	
	5mM Glc	0.85	0.65-1.11		0.11	0.09-0.12		0.23	0.16-0.27	
Inulin 1% (n=10)	50mM Glc	0.50	0.44-0.60	**0.004***	0.05	0.04-0.07	**0.005***	0.20	0.16-0.22	0.426
	100mM Glc	0.98	0.88-1.06		0.06	0.02-0.09		0.20	0.18-0.24	
	5mM Glc	0.98	0.73-1.11		0.07	0.05-0.08		0.18	0.14-0.23	
Inulin 5% (n=10)	50mM Glc	0.97	0.86-1.12	0.52	0.09	0.07-0.12	**0.045***	0.14	0.11-0.17	0.244
	100mM Glc	0.81	0.70-0.91		0.07	0.04-0.10		0.11	0.09-0.21	
	5mM Glc	1,11	1.04-1.20		0.05	0.04-0.05		0.14	0.11-0.17	
GOS 1% (n=10)	50mM Glc	0.71	0.59-0.84	**0.001***	0.16	0.11-0.19	**0.004***	0.18	0.12-0.20	**0.000***
	100mM Glc	1,23	1.17-1.31		0.09	0.04-0.15		0.28	0.25-0.32	
	5mM Glc	0.82	0.74-0.89		0.09	0.07-0.10		0.15	0.13-0.18	
GOS 5% (n=10)	50mM Glc	1,04	0.50-1.29	**0.002***	0.13	0.12-0.16	**0.008***	0.14	0.12-0.20	0.992
	100mM Glc	1,21	1.13-1.30		0.09	0.07-0.12		0.14	0.11-0.23	
	5mM Glc	0.86	0.80-0.94		0.07	0.05-0.07		0.17	0.11-0.18	
FOS 1% (n=10)	50mM Glc	1,09	1.04-1.14	**0.001***	0.12	0.10-0.12	**0.002***	0.22	0.15-0.25	**0.000***
	100mM Glc	1,27	1.20-1.38		0.11	0.10-0.11		0.30	0.25-0.34	
	5mM Glc	1,29	1.18-1.54		0.07	0.06-0.09		0.17	0.12-0.19	
FOS 5% (n=10)	50mM Glc	0.80	0.69-0.89	**0.001***	0.09	0.07-0.11	**0.019***	0.15	0.11-0.20	**0.002***
	100mM Glc	1,02	0.93-1.08		0.11	0.09-0.13		0.25	0.21-0.30	

*statistical significant

When comparing the enzymatic activity of LDH (Table 3) of groups treated with different oligosaccharides with the control group (LDH values above the median of the control 0.95 U/mg protein - Table 1), in the groups with the presence of 5mM glucose, a statistically significant increase was found only in the groups, treated with 5% FOS (p = 0.004). No statistically significant increase was found in the groups with 50mM Glc (p> 0.05). Only three of the groups with the presence of 100mM Glc, showed a statistically significant increase: the groups with 1% GOS (p = 0.012), 5% GOS (p = 0.036) and 1% FOS (p = 0.006).

When comparing the enzymatic activity of HK (Table 3) of groups treated with different oligosaccharides with the control group (HK values above the median of the control 0.01 U/mg protein - Table 1), it was proven that all groups show a statistically significant difference (p<0.05), with the exception of the group with 5mM Glc, when treated with 1% lactulose.

When comparing the enzymatic activity of GR (Table 3) of groups treated with different oligosaccharides with the control group (GR values above the median of the control 0.16 U/mg protein - Table 1), a statistically significant difference was found only in the group with 5mM Glc, when treated with 1% inulin (p=0.037). In the rest of the groups, a statistically significant difference was not found (p>0.05).

In the groups with 50mM Glc, a statistically significant difference was not found anythem. Unlike these groups, in the groups with 100mM Glc a statistically significant difference in the enzyme activity of GR was found in the groups, treated with 1% lactulose (p=0.000), 1% inulin (p=0.018), 1% GOS (p=0.000), 1% FOS (p=0.000) and 5% FOS (p=0.0001). In the rest of the groups' such difference was missing (p>0.05).

From Table 2, it can be seen that a statistically significant difference regarding FRAP in the three subgroups was found in control, as well as in the groups treated with 1% lactulose, 1% inulin, 5% inulin, 1% GOS, 1% FOS and 5% FOS.

Table 2. Descriptive statistics of FRAP (μmol Trolox equivalent) of erythrocytes, treated with oligosaccharides in the presence of 5mM, 50mM and 100mM glucose

		FRAP		
Groups	**Subgroups**	**Median**	**95% CI**	**P (Kruskal- Wallis Test)**
Control (n=10)	5mM Glc	0.99	0.95-1.02	**0.005***
	50mM Glc	1,08	1.02-1.13	
	100mM Glc	1,07	1.02-1.11	
Lactulose 1% (n=10)	5mM Glc	0.87	0.85-0.89	**0.000***
	50mM Glc	1,09	1.08-1.11	
	100mM Glc	0.98	0.97-0.99	
Lactulose 5% (n=10)	5mM Glc	0.77	0.73-0.87	0.196
	50mM Glc	0.86	0.81-0.90	
	100mM Glc	0.82	0.76-0.86	
Inulin 1% (n=10)	5mM Glc	0.75	0.73-0.84	**0.000***
	50mM Glc	1,05	01.1.2011	
	100mM Glc	1,07	1.05-1.08	
Inulin 5% (n=10)	5mM Glc	0.81	0.77-0.87	**0.000***
	50mM Glc	1,04	0.97-1.10	
	100mM Glc	1,12	1.11-1.12	
GOS 1% (n=10)	5mM Glc	1,04	0.95-1.10	**0.000***
	50mM Glc	1,07	1.03-1.09	
	100mM Glc	1,15	1.12-1.21	
GOS 5% (n=10)	5mM Glc	1,11	0.94-1.30	0.608
	50mM Glc	1,15	1.12-1.18	
	100mM Glc	1,17	1.15-1.22	
FOS 1% (n=10)	5mM Glc	1	0.96-1.06	**0.000***
	50mM Glc	1,14	1.13-1.15	
	100mM Glc	1,17	1.14-1.20	
FOS 5% (n=10)	5mM Glc	1,13	1.02-1.18	**0.027***
	50mM Glc	1,07	1.04-1.09	
	100mM Glc	1,12	1.10-1.13	

*statistical significant

When comparing FRAP (Table 3) of groups treated with different oligosaccharides with the control group (FRAP values above the median of the control 0.99 μmol Trolox equivalent - Table 2), a statistically significant increase was found in the groups with the presence of 5mM Glc, treated with 5% FOS (p=0.015). In the groups with the presence of 50mM Glc, a statistically significant increase was found in the groups treated with 1% lactulose, 1% inulin, 1% GOS, 5% GOS, 1% FOS and 5% FOS (p<0.05). In the groups with the most severe hyperglycemia in the presence of 100mM Glc, a statistically significant increase (p<0.05) was found in all groups except for the ones treated with 1% and 5% lactulose.

Table 3. Comparative characteristics between the control group (erythrocytes, treated with 5 mM glucose) and all other groups, examined with Mann-Whitney U test

		LDH (p)	Hexokinase (p)	GR (p)	FRAP (p)
5mM Glc	Lactulose 1%	0.107	0.074	0.148	0.000**
	Lactulose 5%	0.467	**0.004***	0.568	0.000**
	Inulin 1%	0.222	**0.004***	**0.037***	0.000**
	Inulin 5%	0.747	**0.004***	0.469	0.000**
	GOS 1%	0.146	**0.006***	0.111	0.699
	GOS 5%	0.005**	**0.004***	0.209	0.785
	FOS 1%	0.024**	**0.004***	0.237	0.716
	FOS 5%	**0.004***	**0.004***	0.909	**0.015***
50mM Glc	Lactulose 1%	0.107	**0.004***	0.052	**0.000***
	Lactulose 5%	0.004**	**0.004***	0.620	0.000
	Inulin 1%	0.004**	**0.005***	0.068	**0.012***
	Inulin 5%	0.807	**0.004***	0.087	0.163
	GOS 1%	0.004**	**0.004***	0.675	**0.000***
	GOS 5%	0.747	**0.004***	0.404	**0.000***
	FOS 1%	0.332	**0.003***	0.197	**0.000***
	FOS 5%	0.006**	**0.004***	0.223	**0.000***
100mM Glc	Lactulose 1%	0.333	**0.004***	**0.000***	0.536
	Lactulose 5%	0.004**	**0.015***	0.223	0.000**
	Inulin 1%	0.872	**0.013***	**0.018***	**0.000***
	Inulin 5%	0.012**	**0.005***	0.130	**0.000***
	GOS 1%	**0.012***	**0.035***	**0.000***	**0.000***
	GOS 5%	**0.036***	**0.004***	0.383	**0.000***
	FOS 1%	**0.006***	**0.004***	**0.000***	**0.000***
	FOS 5%	0.747	**0.004***	**0.001***	**0.000***

* statistically significant increase of the respective indicator relative to control- erythrocytes, treated with 5mM Glc
** statistically significant decrease of the respective indicator relative to control- erythrocytes, treated with 5mM Glc

DISCUSSION

Hyperglycemia causes various changes in structure, functions and longevity of erythrocytes. These changes could be due to oxidative stress, which leads to an increase in lipid peroxidation. This peroxidation and the accumulation of malondialdehyde can lead to a disruption of the organization of the phospholipid bilayer and hence the inability of glucose intake from erythrocytes [27]. From table 1 and table 3, it can be seen that under the condition of hyperglycemia in the presence of all oligosaccharides, there is an increase in the activity of the initial enzyme, which activates glucose in erythrocytes, precisely HK. High concentration of glucose can cause posttranslational modification in the structure of proteins which serve as an enzymes and that is why some grups showed a decrease in the enzyme activity of HK after treatment with 100mM of glucose comparing with those treated with 50mM of glucose. It can be assumed that there is no competition between the various oligosaccharides and glucose for GLUT1, and it enters erythrocytes unimpeded. We cannot say if or how oligosaccharides additionally activate HK, but we can say that in the condition of hyperglycemia, there is not inhibition of HK.

It is known that the erythrocytes of patients with diabetes have structural and functional changes. Altered concentrations of glycolytic chain metabolites, as well as accumulation of lactate due to increased activity of LDH, are also observed [18]. Our results confirm the literature data, as a statistically significant increase in the activity of LDH was found under the condition of hyperglycemia, caused by the presence of 100mM Glc, in erythrocytes, treated with 1% GOS (p=0.012), 5% GOS (p=0.036) and 1% FOS (p=0.006). It is an interesting fact that in some of the groups, a statisti-

cally significant decrease in the activity of LDH was observed relative to the median of the control. In the condition of hyperglycemia, caused by 50mM Glc, a decrease of LDH was observed in the following groups: 5% lactulose (p=0.004), 1% inulin (p=0.004), 1% GOS (p=0.004) and 5% FOS (p=0.006). In the groups with the presence of 100mM Glc, a statistically significant decrease of LDH was observed in the following groups: 5% lactulose (p=0.004) and 5% inulin (p=0.012) (Table 3). Our explanation is that the decrease of LDH in these groups may be due to the redirection of metabolites towards the pentose phosphate pathway because of the increased oxidative stress under the conditions of hyperglycemia and the activation of antioxidant enzymes which protect erythrocytes. This is partially supported by our results on the activities of enzymes involved in the antioxidant protection of these cells.

Glutathione is an important intracellular antioxidant, which is maintained in a reduced state through the pentose phosphate pathway, which uses 3-11% of the glucose in erythrocytes. This is the only pathway, which generates NADPH needed for the decrease of the oxidized glutathione (GSSG) [15] via the action of GR [20]. In literature, there are contradictory data regarding the activity of GR in conditions of hyperglycemia. Viskupicova et al. have established that under in vitro conditions of hyperglycemia, there is a decrease in the activities of GR and GST [15]. We obtained a statistically significant increase in the activity of GR under the condition of severe hyperglycemia (100mM Glc) after treatment with the following oligosaccharides: 1% lactulose, 1% inulin, 1% GOS, 1% FOS and 5% FOS ($p<0.005$) (Table 3). This can be due to the eventual influence of these oligosaccharides on the membrane protein systems Syk, Lyn and band 3. These systems bind some of the enzymes of erythrocytes and distribute the use of glucose in a balanced way, both in glycolysis and in the pentose phosphate pathway. Under the conditions of hyperglycemia, oligosaccharides decrease the rate of glycolysis and activate the production of NADPH in the pentose phosphate pathway and, in this way, enhance the protective mechanisms of the erythrocyte [16].

Because of the increased production of reactive oxygen species under conditions of hyperglycemia, the total antioxidant capacity of erythrocytes increases in the presence of various oligosaccharides, which can be seen from the data about FRAP (Table 3). A correlation between GR and FRAP was not established, which may be due to the activation of some other antioxidant enzymes by oligosaccharides, which actually lead to an increase in total antioxidant capacity.

CONCLUSION

Due to the lack of data in the literature on in vitro experiments on erythrocytes treated with oligosaccharides, we offer a new perspective on changes in glycolytic chain enzymes and antioxidant protection. The mechanisms by which oligosaccharides affect enzymes are still unknown to us.

GOS 1%, FOS 1% and FOS 5% are these oligosaccharides, which caused a statistically significant increase of the enzymatic activities of the hexokinase and glutathione reductase in in vitro hyperglycemia induced by 100 mM Glc, as well as an increase in FRAP. Lactate dehydrogenase was decreased in the presence of 5% lactulose in groups with 50 mM and 100 mM Glc. These results predetermine further studies on other models of in vitro and in vivo treatment with oligosaccharides and study of their positive effects on the various metabolic pathways in the human body and elucidation of the mechanisms that lead to them.

Abbreviations:

SCFAs – short chain fatty acids;
GPCR – G-protein coupled receptors;
IL – interleukin;
HDAC – histone deacetylase;
GLP-1 – glucagon like peptide 1;
LPS – lipopolysaccharides;
FOS – fructooligosaccharides;
GOS – galactooligosaccharides;
XOS – xylooligosaccharides;
MOS – manooligosaccharides;
HMO – oligosaccharides from human milk;
DM – diabetes mellitus;
IDF – International Diabetes Federation;
ATP – adenosine triphosphate;
NADPH – reduced nicotinamide adenine dinucleotide phosphate;
HK – hexokinase;
PFK – phosphofructokinase;
PK – pyruvate kinase;
LDH – lactate dehydrogenase;
SOD – superoxide dismutase;
CAT – catalase;
GST – glutathione S-transferase;
GR – glutathione reductase;
GSSG – oxidized glutathione;
GSH – reduced glutathione;
CI – confidence interval
Glc – glucose;
FRAP – ferric reducing antioxidant power

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