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DIETARY SUPPLEMENTATION WITH MAGNESIUM CITRATE MAY IMPROVE PANCREATIC METABOLIC INDICES IN AN ALLOXAN-INDUCED DIABETES RAT MODEL

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ABSTRACT

The purpose of the current study was to evaluate the protective properties of dietary magnesium supplementation on pancreatic tissue of rats with alloxan-induced *diabetes mellitus*. Twenty-five male Wistar rats were split into five groups (control, diabetes, diabetes with 100 mg Mg daily, diabetes with 250 mg Mg daily, diabetes with 500 mg Mg daily) with feeding supplementation starting on day 1, diabetes induction on day 21, and animal sacrifice on day 30. Fasting glucose in blood serum was measured on days 21, 25, 27, and day 30. Glucose metabolism enzymes, namely, lactate dehydrogenase and glucose-6-phosphate dehydrogenase, were measured in pancreatic tissue upon the sacrifice, as well as lipid peroxidation, antioxidant system protective enzymes (catalase and superoxide dismutase), and glutathione system components (glutathione reductase, glutathione peroxidase, and glutathione reduced). Pearson correlation coefficients showed strong negative correlation between serum glucose (control and diabetic animals) and glucose metabolism enzymes, catalase, superoxide dismutase, glutathione peroxidase in pancreatic tissue ($r > -0.9$, $p < 0.05$), moderate negative correlation with reduced glutathione ($r = -0.79$, $p < 0.05$), moderate positive correlation with lipid peroxidation index ($r = +0.67$, $p < 0.05$), weak correlation with glutathione reductase ($r = -0.57$, $p < 0.05$). Magnesium supplementation slowed down diabetes onset considering fasting glucose levels in rats ($p < 0.05$), as well as partially restored investigated dehydrogenase levels in the pancreas of rats comparing to diabetes group ($p < 0.05$). The lipid peroxidation index varied between treatments showing the dose-dependent influence of Mg^{2+}. Magnesium supplementation partially restored catalase and superoxide dismutase activities in pancreatic tissue, as well as glutathione peroxidase and reduced glutathione levels ($p < 0.05$), while glutathione reductase levels remained unaffected ($p > 0.05$). The obtained results suggested a model, where magnesium ions may have a possible protective effect on pancreatic tissue against the negative influence of alloxan inside β cells of the pancreas.

Keywords: alloxan; diabetes; rat model; magnesium oral supplementation; pancreas

INTRODUCTION

Diabetes mellitus is a major health-related problem worldwide, with the number of people with diabetes increasing from 108 million in 1980 to 422 million in 2014, according to the WHO **(WHO, 2018)**. Dietary habits, together with a genetic predisposition, may influence *diabetes mellitus* development in healthy individuals. Magnesium is a macroelement involved in virtually all biochemical pathways in cells **(de Baaij, Hoenderop and Bindels, 2015)**. Considering its importance, the serum Mg^{2+} levels are maintained between 0.7 and 1.05 mM level in blood serum **(de Baaij, Hoenderop and Bindels, 2015)**. Low serum Mg^{2+} levels could be attributed to either dietary restriction, impaired intestinal absorption, or increased renal wasting. Hypomagnesemia has been long reported as a risk factor for type 2 *diabetes mellitus* development, as a contributing reason for **(Dong et al., 2011)**, as well as a consequence of insulin resistance **(Pham et al., 2007; Chaudhary, Sharma and Bansal, 2010)**. "A vicious cycle" of hypomagnesemia described by **Gommers et al. (2016)** implies the necessity of Mg^{2+} for insulin receptor autophosphorylation and therefore preventing insulin resistance from one side, while circulating excess of insulin being able to activate renal Mg^{2+} channel transient receptor potential melastatin type 6, which stimulates the secretion of Mg^{2+} with urine. Magnesium influences glucose metabolism and insulin action in several ways. Firstly, it is involved in the autophosphorylation of β-subunits of insulin receptor through tyrosine kinase activity binding to ATP. Rats with hypomagnesemia had reduced levels of the phosphorylated insulin receptor, mimicking insulin resistance **(Paxton and Ye, 2005; Suárez et al., 1995)**. Secondly, recent studies showed that

oral Mg^{2+} supplementation increased GLUT1 and GLUT4 expression in muscles of STZ-induced diabetic rats and mice, resulting in lowering serum glucose levels and its uptake by muscle tissue **(Biddinger and Kahn, 2006; Solaimani et al., 2014)**. Thirdly, Mg^{2+} is an important anti-inflammatory mediator, and hypomagnesemia increased production of proinflammatory interleukin 1, tumor necrosis factor-α by adipocytes, causing the production of reactive oxygen species that may lead to chronic inflammation, insulin resistance, and reduced GLUT4 activity **(Rodriguez-Moran and Guerrero-Romero, 2004; Weglicki et al., 1992)**. And finally, Mg^{2+} affects insulin production in pancreatic β cells. Under normal physiological conditions, increased blood glucose levels stimulate the influx of glucose into β-cell via GLUT2 transporter, followed by its conversion to glucose-6-phosphate (G6P) by glucokinase, which is a " glucose sensor" for insulin secretion. G6P is further metabolized through glycolysis and Krebs cycle, producing ATP, excess of which directly induces closure of K_{ATP} channel Kir6.2. Closure of this channel causes depolarization of plasma membrane, the opening of a voltage-dependent influx of Ca^{2+} ions, which causes exocytosis of insulin into the bloodstream. From one side, Mg^{2+} directly positively influences glucokinase activity by acting as a cofactor of adenine nucleotides, though it may be happening even at sub-physiological levels. Conversely, Mg^{2+} initiates K_{ATP} channel opening through MgATP, negatively affecting L-type Ca^{2+} channels, an influx of Ca^{2+} into β cells, and restraining insulin release. However, those are short-time negative consequences, while in a long run Mg^{2+} may improve β cells functionality, similar to cardiomyocytes, where low Mg^{2+} level was shown to decrease expression of L-type Ca^{2+} channels **(Gommers et al., 2016)**. It was shown that in humans without diabetes decreased serum Mg^{2+} was associated with decreased insulin secretion **(Rodríguez-Morán and Guerrero-Romero, 2011)**. Similarly, supplementation of individuals without diabetes with $MgCl_2$ significantly improved β-cell function and insulin secretion **(Guerrero-Romero and Rodríguez-Morán, 2011)**.

Alloxan is a cytotoxic glucose analog, which selectively binds to GLUT2 receptors of pancreatic β cells and enters them, causing necrotic cell death, thus modeling type 1 diabetes **(Lenzen, 2008)**. Alloxan is highly unstable in solutions with neutral pH and bloodstream and decomposes to alloxanic acid within minutes **(Szkudelski, 2001)**. A single intraperitoneal injection of 150 $mg.kg^{-1}$ of live weight is sufficient to cause alloxan-induced diabetes in fasting rats, causing permanent hyperglycemia between 24 to 48 hours post-injection **(Ighodaro, Adeosun and Akinloye, 2017)**. Being a thiol reagent, alloxan inhibits glucokinase ("glucose sensor"), and, therefore, glucose phosphorylation in β cells, initially causing rapid ATP increase followed by rapid insulin secretion and severe hypoglycemia, followed by halting and inhibition of insulin secretion **(Szkudelski, 2001; Lenzen, 2008; Ighodaro, Adeosun and Akinloye, 2017)**. More importantly, alloxan enters redox cyclic reaction coupled with its oxidized form, dialuronic acid, which spontaneously autooxidizes to generate toxic superoxide radicals and hydrogen peroxide, commonly known as reactive oxygen species (ROS) **(Munday, 1988; Winterbourn and Munday, 1989; Lenzen, 2008)**. Thiol groups, specifically, but not limited, to reduced glutathione are required for dialuronic acid production **(Szkudelski, 2001)**. A temporary compound of alloxan-glutathione, namely, "compound 305", is formed during each cycle, resulting in decreased GSH/GSSG ratio and depletion of reduced glutathione **(Munday, 1988; Szkudelski, 2001)**. The ultimate damage is caused by another ROS, hydroxyl radicals, which acts together with superoxide radicals and hydrogen peroxide, causing massive cellular damage, and ultimately apoptosis. Interestingly, few reviews noted massive Ca^{2+} influx in β cells and subsequent disruption of intracellular calcium homeostasis as one of the major reasons for β cell necrosis as well **(Szkudelski, 2001; Ankur and Shahjad, 2012)**. Antioxidant enzymes, namely, superoxide dismutase (transforming superoxide radical into hydrogen peroxide), and catalase (transforming hydrogen peroxide into water and oxygen), protect cells against ROS produced by alloxan **(Grankvist et al., 1979; Winterbourn and Munday, 1989)**. Glutathione peroxidase also detoxifies cells from hydrogen peroxide, though it requires reduced glutathione as a substrate. Cell debris, produced by necrotic dying β cells, are quickly scavenged by macrophages.

The purpose of the current study was to evaluate the influence of magnesium dietary supplementation on the duration and late progression of alloxan-induced diabetes related to pancreatic metabolic indices, including enzymes of glucose metabolism (lactate dehydrogenase, LDH, and glucose-6-phosphate dehydrogenase, G6PD), state of lipid oxidation (lipid hydroperoxide, LOOH), antioxidant enzymes (catalase, CAT, and superoxide dismutase, SOD), and glutathione system (glutathione peroxidase, G-Per, glutathione reductase, G-Red, and reduced glutathione, GSH). To the best of our knowledge, this study relative to pancreatic health state after late progression of alloxan-induced diabetes, with or without oral magnesium supplementation, was not performed yet. Additionally, we measured fasting serum glucose levels in rats with alloxan-induced diabetes, with or without dietary magnesium supplementation, as a time-dependent variable influenced by diabetes onset and progression.

Scientific hypothesis

We hypothesize that magnesium supplementation will not prevent, but may reduce the toxic effects of alloxan on pancreatic β cells. This may include the restoration of pancreatic glucose metabolism enzyme function back to the levels of the healthy control rats; decrease in LOOH comparing to alloxan-treated rats, reducing their levels back to the levels found in healthy control rats; activation of CAT and SOD, as protectors against ROS; increase in levels of G-SH and glutathione system enzymes, namely, G-Red and G-Per. Additionally, we hypothesize that magnesium supplementation may reduce fasting blood glucose levels in alloxan-induced diabetic rats due to the improved pancreatic function.

MATERIALS AND METHODOLOGY

The alloxan-induced diabetes rat model

Twenty-five white Wistar rats (ca. 140 g each, same age) were used for the experiments. The animals were kept in cages under standard conditions (20 – 25 °C, 40 – 45% air relative humidity) with free access to compound feed, while water was strictly supplemented (20 mL daily). Compound feed composition remained unknown. All procedures with animals were carried out according to the "European Convention for the Protection of Vertebrate Animals for Experimental and Other Scientific Purposes" (Strasbourg, France, Council of Europe, March 18, 1986) and were approved by the local institutional ethical committee.

The rats were divided into five groups, 5 rats per group: intact control (group 1, C); induced diabetes (group 2, D); magnesium supplementation (100 mg Mg^{2+} kg^{1} weight, daily dose) with diabetes (group 3, Mg100-D); magnesium (250 $mg.kg^{-1}$) with diabetes (group 4, Mg250-D); magnesium (500 $mg.kg^{-1}$) with diabetes (group 5, Mg500-D). Day 0 was the date of dietary supplementation start, while alloxan induction of diabetes was performed on day 21, and all animals were sacrificed on day 30. Magnesium citrate ($C_6H_6O_7Mg$) was dissolved in tap water in the amount of 6.17, 15.44, and 30.88 mg per mL corresponding to 14, 35, or 70 mg Mg^{2+} in 20 mL water per animal, which relates to 100, 250, and 500 mg Mg^{2+} per kg of live weight. A single intraperitoneal injection of alloxan monohydrate ("Sinbias", Ukraine) in 0.85% physiological saline was performed on each rat from groups 2, 3, 4, and 5 (150 $mg.kg^{-1}$ of body weight) after 24-hours fasting on the 21st day from the start of the experiment. All animals were sacrificed under ether anesthesia by decapitation on day 30, followed by removal of the pancreas within 2 minutes after the death. The removed pancreas was kept on ice for 5 to 6 minutes followed by bleeding through repeated perfusion with physiological saline (0.85% NaCl, 4 °C). The pancreas was chopped coarsely by scissors. One gram of chopped tissue was homogenized with 9.0 mL of 5 mM Tris-HCl buffer (1:10 dilution), pH 7.4, for 50 seconds at 0 °C (MRTU-421505-63, Ukraine). The homogenate was filtered through two layers of cheesecloth into a centrifuge tube followed by centrifugation (3,000 g, 10 minutes, 0 °C). The supernatant was used immediately for biochemical markers quantification and called "tissue sample" throughout the research paper.

All reagents used for the experiments, if not specified, were of laboratory grade or better.

Fasting blood serum glucose determination

Glucose determination was done on venous blood drawn from rat tails using glucose oxidase method (Gamma TM mini glucometer) after at least 6-hours fasting on days 21, 23, 25, 27, and day 30 (sacrifice day). The principle of the method is based on the reaction catalyzed by glucose oxidase:

$$\text{Glucose} + O_2 \rightarrow \text{Glucolactone} + H_2O_2 \quad (1)$$

using test strips and calibration strips supplied by the manufacturer.

Lipid hydroperoxide (LOOH) determination

LOOH determination is based on the interaction of ethanol extracts of lipid hydroperoxides with ammonium thiocyanate after precipitation of proteins with trichloroacetic acid (TCA). The original method developed by **Wagner, Clever and Peters (1947)**, and later updated and modified by **Mihaljević, Katušin-Ražem and Ražem (1996)**, was adapted. Briefly, 0.2 mL of tissue sample was placed in a centrifuge tube with 2.8 mL of ethanol (95%) and 0.05 mL of 50% TCA at 4 °C. The tube was tightly closed and shaken for 5 minutes, followed by centrifugation, resulting in protein precipitation (3,000 g, 10 minutes, 4 °C). 1.5 mL of the resulting supernatant was mixed with 1.2 mL of ethanol, 0.02 mL of concentrated HCl, and 0.03 mL of 1% Mohr's salt, $Fe(NH_4)_2(SO_4)_2$, in 3% HCl. The mixture was shaken, followed by addition of 0.2 mL of 20% NH_4SCN. Optical absorbance (A_{480}) was measured after 10 minutes of incubation time (spectrophotometer UNICO S1205, λ = 480 nm). The control sample was prepared by tissue sample replacement with 0.2 mL of deionized water. LOOH in samples was calculated using the following equation:

$$\Delta A_{480}\ \text{LOOH}.g^{-1} = DF \times (A_{480\ exp} - A_{480control}) \quad (2)$$

The obtained results were expressed as the difference in optical absorbance between experimental and control samples (λ = 480 nm), adjusted to 1 g of pancreatic tissue (ΔA480 $\text{LOOH}.g^{-1}$) by dilution factor DF.

All reagents used for the experiments, if not specified, were of laboratory grade or better.

Total protein determination

Protein concentration in the tissue sample was determined by the Lowry method using standardized kits (SIMCO Ltd, Ukraine) according to the manufacturer's instructions. The method is based on the formation of non-ferrous products of the interaction of aromatic amino acids with the Folin-Ciocalteu reagent in combination with the biuret reaction to peptide bonds of the protein **(Lowry et al., 1951)**. The results were expressed in mg of protein per mL of the tissue sample using spectrophotometer UNICO S1205 for absorbance measurements. The protein concentration of tissue samples from the pancreas of each rat was used to estimate enzyme activity per mg of protein in the subsequent analysis of SOD, CAT, G-Per, G-Red, G6PD, and LDH enzymatic activities in tissue samples.

Superoxide dismutase (SOD) determination

The principle of the method for superoxide dismutase (EC 1.15.1.1) activity determination is based on the ability of the enzyme to compete with nitroblue tetrazolium for the superoxide anion radicals, which are formed as a result of aerobic interaction of NADH and phenazine methosulfate **(Ponti et al., 1978)**. As a result of this reaction, nitroblue tetrazolium is reduced to formazan dye. In the presence of SOD, the percentage of nitroblue tetrazolium reduction is reduced. Enzyme activity was determined by the percentage of blockage of formazan dye formation **(Durak et al., 1993)**. Briefly, 1.5 mL of incubation mixture (containing 37 mg EDTA-Na_2, 330 mg nitrotetrazolium blue, 55 mg phenazine methosulfate, and

0.3 mL 0.15 M phosphate buffer, pH 7.8) was mixed with 0.1 mL of a tissue sample and 0.1 mL of 1 mM NADH in Tris-EDTA buffer (pH 8.0) followed by 10 minutes incubation at room temperature. Optical absorbance was measured against water (UNICO S1205, $\lambda = 540$ nm). The control sample was prepared using distilled instead of a tissue sample. The enzyme activity was determined with the following equation:

$$\% \text{ blockage} = 100 \times (A_c - A_t)/A_c \quad (3)$$

where A_c – optical absorbance of the control sample; A_t – optical absorbance of the test sample. Superoxide dismutase activity was expressed in relative units per 1 mg of protein (1 relative unit = 50% blocking).

Catalase (CAT) determination

The principle of the method for catalase (EC 1.11.1.6) activity determination is based on the ability of H_2O_2 to form a stable colored complex with molybdenum salts. According to **Góth (1991)**, the color intensity of molybdenum peroxide compounds depends on the amount of H_2O_2 in solution; and therefore, on the activity of catalase in the sample. The catalase reaction was started by mixing 0.1 mL of a tissue sample with 1 mL of 0.05 M Tris-HCl buffer (pH 7.8) and 2 mL of 0.03% H_2O_2. Blank included 1 mL of 4% $(NH_4)_2MoO_4$ in 0.025 N H_2SO_4 and 2.0 mL H_2O_2. The reaction in the test sample was stopped after 10 minutes by adding 1 mL of 0.25 N H_2SO_4 and 1 mL of 4% $(NH_4)_2MoO_4$. To complete the blank, 1 mL of 0.25 N H_2SO_4 and 0.1 mL of tissue sample was added to the blank.

Samples were centrifuged for 5 minutes at 3,000 g. Optical absorbance was determined spectrophotometrically (UNICO S1205, $\lambda = 410$ nm). The catalase activity in $\mu mol.min^{-1}.mg^{-1}$ was calculated using the following equation:

$$E_a = (\Delta A \times DF)/(\varepsilon \times t \times C \times l) \quad (4)$$

Where: ΔA – difference between the optical absorbance of blank and test samples; DF – dilution factor of the original tissue sample; ε – molar extinction coefficient of the H_2O_2 complex with ammonium molybdate at $\lambda = 410$ nm, equal to 22200 $M^{-1}.cm^{-1}$; C – tissue sample protein concentration, $mg.mL^{-1}$; t – reaction time, 10 minutes, l – optical path, 1 cm.

All reagents used for the experiments, if not specified, were of laboratory grade or better.

Glutathione peroxidase (G-Per) determination

The activity of glutathione peroxidase (EC 1.11.1.9) was measured through the oxidation rate of reduced glutathione in the presence of tertiary butyl hydroperoxide **(Moin, 1996)**. Change in absorbance is due to the interaction between SH-groups and Ellman's reagent (0.01 M solution of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in methanol) with the formation of a colored product, 5'-thio-2-nitrobenzoic acid. The amount of the latter is directly proportional to the number of -SH groups that reacted with Ellman's reagent. Briefly, 0.83 mL of 4.8 mM reduced glutathione in 0.1 M Tris-HCl buffer (pH 8.5), containing 6 mM EDTA and 12 mM NaN_3, was added to 0.2 mL of the tissue sample. The mixture was incubated at 37 °C for 10 minutes. Following incubation, 0.07 mL of 20 mM tertiary butyl hydroperoxide was added and the mixture was incubated for an additional 5 minutes. The reaction was stopped by adding 0.2 mL of 20% trichloroacetic acid at 4 °C. A blank sample was prepared identically, but with distilled water instead of the tissue sample. The samples were centrifuged for 10 minutes at 3000 g. The supernatant (0.02 mL) was mixed with 2 mL of 0.1 M Tris-HCl buffer (pH 8.5) and 0.02 mL of Elman's reagent. After 5 minutes incubation, the samples were analyzed spectrophotometrically (UNICO S1205, $\lambda = 412$ nm). The G-Per result was expressed in $\mu mol.min^{-1}.mg^{-1}$ protein and calculated using the following equation:

$$E_a = (\Delta A \times DF \times C_s)/(A_s \times t \times C) \quad (5)$$

Where: ΔA – the difference between optical absorbance of the control and experimental samples; DF – dilution factor of the original tissue sample; A_s – absorbance of the standard G-SH solution; C_s – concentration of the standard G-SH solution; C – protein concentration in the tissue sample, $mg.mL^{-1}$; t – reaction time, 5 minutes.

Glutathione reductase (G-Red) determination

The activity of glutathione reductase (EC 1.8.1.7) was determined spectrophotometrically as the rate of glutathione reduction in the presence of NADPH as suggested by **Carlberg and Mannervik (1985)**. The reaction mixture contained 2 mL of 0.15 M phosphate buffer, pH 7.4; 0.2 mL of 10 mM EDTA**;** 0.5 mL of 7.5 mM oxidized glutathione, 0.1 mL of 2 mM NADPH; and 0.2 mL of the test sample. The enzyme activity was determined by measuring the reduction in NADPH content within 10 minutes at 37 °C (UNICO S1205, λ =340 nm). The activity of G-Red was expressed in $\mu mol(NADPH).min^{-1}.mg^{-1}$ protein and calculated using equation 4, where ΔA – absorbance difference between the start and end time of the reaction; DF – dilution factor of the tissue sample; ε – molar extinction coefficient for NADPH, $\varepsilon = 6220\ M^{-1}.cm^{-1}$; t – reaction time, 10 minutes.

Reduced glutathione (G-SH) determination

The content of reduced glutathione (G-SH) was determined by the level of 5'-thio-2-nitrobenzoic acid formation as a result of the interaction of the glutathione – SH groups with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) at $\lambda = 412$ nm as per method developed by **Beutler, Duron and Kelly (1963)**. The following reagents were used: a precipitating reagent (glacial metaphosphoric acid – 6.68 g; EDTA – 0.80 g; sodium chloride – 120.0 g; distilled water to 400 mL); 0.3 M Na_2HPO_4 solution in distilled water; Elman's reagent (0.04% solution of 5.5-dithiobis-2-nitrobenzoic acid in 1% solution of trisodium citrate) **(Rahman, Kode and Biswas, 2006)**.

Stage I. Sample: tissue sample – 2 mL, precipitating reagent – 3 mL; control: distilled water – 2 mL, precipitating reagent – 3 mL. The mixtures were kept at room temperature for 5 minutes followed by centrifugation

at 3,500g, through which protein-free supernatant was obtained.

Stage II. Sample: supernatant from stage I – 2 mL, 0.3 M Na_2HPO_4 – 8 mL, Elman's reagent – 0.1 mL; Control: control supernatant from stage I – 2 mL; 0.3 M Na_2HPO_4 – 8 mL, Elman reagent– 0.1 mL. After 5 minutes of incubation, the absorbance measurement of the sample against control was performed (UNICO S1205, λ = 412 nm). The calculation of the amount of G-SH in the pancreas in mmoles per gram of tissue was estimated against G-SH standard solution with known concentration C_s using equation 6:

$$N_{GSH} = (\Delta A_{412} \text{ x } DF \text{ x } C_s)/A_{412s} \quad (6)$$

All reagents used for the experiments, if not specified, were of laboratory grade or better.

Lactate dehydrogenase (LDH) and glucose-6P-dehydrogenase (G6PD) determination

LDH and G6PD enzymatic activity were determined using spectrophotometric methods based on the coupled oxidation/reduction systems of nicotinamide coenzymes (UNICO S1205, 37 °C, NADH and NADPH molar extinction coefficient ε = 6220 at λ = 340 nm, t = 5 minutes of enzymatic reaction). The reaction was carried in 0.2 M tris-HCl buffer (pH 7.5) with a total volume of 3 mL, including 0.2 mL of the tissue sample. The final concentrations of the reaction components were as follows:

LDH – 1×10^{-3} M sodium pyruvate, 5×10^{-5} NADH, 3×10^{-3} M $MgCl_2$ **(Sevela and Tovarek, 1959)**.

G6PD – 1×10^{-3} M glucose-6-phosphate, 5×10^{-3} M $MgCl_2$; 0.5×10^{-4} M $NADP^+$ **(Evans, 2009)**.

The enzymatic activity was expressed in nmole.min^{-1}.mg^1 protein and calculated using equation 4.

All reagents used for the experiments, if not specified, were of laboratory grade or better.

Statistical analysis

Two-factorial ANOVA was used to analyze the influence of treatment (D, Mg100-D, Mg250-D, Mg500-D) and time of experiment onset (day 21, day 25, day 27, and day 30), as well as their interaction (treatment x time), on the fasting glucose levels in rats. If the influence of factors or their interaction was significant (p <0.05), means were separated using the Fisher LSD procedure.

Pearson correlation coefficients were calculated between paired serum glucose levels of the control (C) and diabetes-induced rats (D) on day 30 and LOOH, SOD, CAT, G-Per, G-Red, G-SH, G6PD, and LDH levels in pancreas after animal sacrifice.

One-way ANOVA was used to analyze the influence of treatment (C, D, Mg100-D, Mg250-D, Mg500-D) on carbs metabolism enzyme activity, namely, G6PD and LDH; on lipid oxidative damage index, namely, LOOH level; on antioxidant enzymes activity, namely, SOD and CAT; and on glutathione system, namely, G-Per and G-Red activities and G-SH levels, in pancreatic tissue. If the influence of the factor was significant (p <0.05), means were separated using the Fisher LSD procedure.

RESULTS AND DISCUSSION

As expected, oral magnesium supplementation did not prevent alloxan induction of diabetes, though somewhat significantly slowed its progression (Figure 1, p <0.05). There was a significant influence of both factors, treatment (D, Mg100-D, Mg250-D, Mg500-D) and time of experiment onset (day 21, day 25, day 27, and day 30), on fasting serum glucose concentration (p <0.05, Figure 1), though their interaction (treatment x time) did not affect (p >0.05). Glucose levels increased gradually with the time of the experiment onset (Figure 3), while 250 $mg.kg^{-1}$ magnesium-dose had the most profound effect on the fasting glucose reduction (Figure 2). Similarly, **Abayomi et al. (2011)** showed that a single intraperitoneal injection of magnesium (100 and 150 $mg.kg^{-1}$ live weight) one hour before alloxan-induced diabetes (120 $mg.kg^{-1}$ live weight) in rats improved fasting glucose levels on days 2, 5, 7, and 10 compared to diabetes alone, but still was significantly higher comparing to control. Additionally, **Ige et al. (2010)** showed a similar effect of a single intraperitoneal injection of magnesium (100 $mg.kg^{-1}$) one hour before diabetes induction in rats even at a higher level of alloxan dose (150 $mg.kg^{-1}$ live weight).

The Pearson correlation coefficients between serum glucose levels (C and D groups) and investigated indices are shown in Table 1. The increased glucose levels, as a result of alloxan-induced diabetes, had strong negative correlations with LDH and G6PD enzymes, possibly indicating cellular damage and reduced metabolic activity of pancreatic cells; strong negative correlations with CAT, SOD, G-Per, possible indicating damage of antioxidative enzyme system as a result of confronting ROS produced by alloxan; medium negative correlations with G-SH, indicating G-SH consumption and possible impairment during alloxan redox cycling with dialuronic acid. A weak negative correlation was observed with G-Red, and moderate positive correlation with LOOH, indicating residual oxidative damage to the lipids of the cell membranes. These weak positive correlations could be because alloxan-induced diabetes was already established and necrotic β cells already removed by macrophages before the day of animal sacrifice (day 30).

Polyunsaturated fatty acids (PUFAs), which in their esterified state are present in membrane or storage lipids, are prone to ROS-induced peroxidation, resulting in the damage to the biomembranes. Final products of lipid peroxidation (LOOH) are reactive aldehydes, which are relatively stable, can diffuse far from the location of oxidative injury, and may act as second messengers or free radicals **(Gasparovic et al., 2013)**. Levels of LOOH were significantly different between treatments as shown in Table 2 (p <0.05). LOOH increased non-significantly in the diabetes group, and was significantly decreased compared to diabetes in Mg250-D group (p <0.05). Paradoxically, the highest LOOH levels were observed at high Mg^{2+} supplementation (500 $mg.kg^{-1}$), possibly showing that at such levels influence of Mg^{2+} may be negative, which is also confirmed by increased fasting serum glucose in this group (Figure 2). Though the major effect of targeted alloxan cytotoxic action on β cells is massive production of ROS, such as superoxide radical, hydrogen peroxide, and hydroxyl radical **(Munday, 1988; Winterbourn and Munday, 1989)**, the result is apoptosis

of β cells followed by cell debris removal. The determination of LOOH was done long after alloxan major damage and after its degradation in rat body (day 10 after the onset of diabetes), and presumably all damaged cells were either removed or repaired back to normal. Therefore, LOOH values could be non-informative.

Another possibility is the lack of the precision in determination of these values due to the low number of observations, causing an absence of significant differences between treatments.

Evaluation of enzyme activity, due to their protein nature, and sensitivity to various processing conditions, as well as pathological processes, is widely used in industry, life science, and medical research **(Tokarskyy et al., 2009; Yaremchuk and Posokhova, 2011; Lykhatskyi et al., 2019)**.

Table 1 Pearson correlation coefficients (p <0.05) between fasting serum glucose levels in control (C) and diabetic (D) groups vs. investigated indices (LOOH, SOD, CAT, G-Per, G-Red, G-SH, LDH, G6PD).

	LOOH	SOD	CAT	G-per	G-red	G-SH	LDH	G6PD
Pearson correlation coefficient, r	+0.67	-0.92	-0.98	-0.91	-0.57	-0.79	-0.93	-0.95

Table 2 Influence of treatment (control, diabetes, diabetes with 100, 250, and 500 mg Mg supplementation) on LOOH levels in pancreatic tissue of rats upon sacrifice on day 30, with standard deviation included.

	C ±SD	D ±SD	Mg100-D ±SD	Mg250-D ±SD	Mg500-D ±SD
LOOH, $\Delta A_{480}.g^{-1}$	0.133 ±0.014 bd*	0.165 ±0.021 b	0.143 ±0.014 bc	0.133 ±0.013 cd	0.210 ±0.011 a

Note: *Means with the same letters are not significantly different (p >0.05).

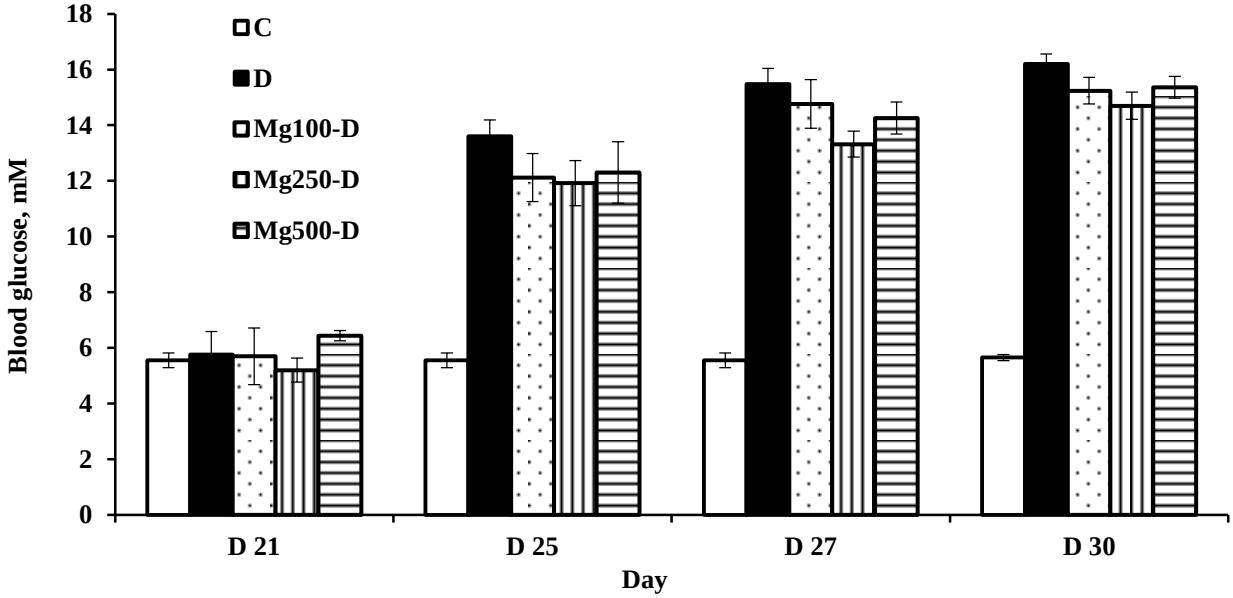

Figure 1 Mean values of fasting blood serum glucose levels in control (C), diabetic (D), magnesium supplemented diabetic rats (Mg100-D, Mg250-D, Mg500-D) as measured on days 21, 25, 27, and sacrifice day (day 30). Control (C) is shown for reference. Error bars reflect standard deviations. Note: Means with the same letters are not significantly different (p >0.05).

Table 3. Influences of treatment (control, diabetes, diabetes with 100, 250, and 500 mg Mg supplementation) on G-Per (p <0.05), G-Red (p =0.7), and G-SH (p <0.05) levels in pancreatic tissue of rats upon sacrifice on day 30, with standard deviation included.

	C ±SD	D ±SD	Mg100-D ±SD	Mg250-D ±SD	Mg500-D ±SD
G-Per, $\mu mol(G\text{-}SH).min^{-1}.mg^{-1}$	3.30 ±0.43 ab*	1.80 ±0.14 c	2.84 ±0.31 b	3.97 ±0.42 a	2.44 ±0.28 bc
G-Red, $\mu mol(NADPH).min^{-1}.mg^{-1}$	0.028 ±0.001 a*	0.024 ±0.002 a	0.025 ±0.002 a	0.026 ±0.005 a	0.024 ±0.004 a
G-SH, $mmol.g^{-1}$	0.031 ±0.008 a*	0.015 ±0.005 b	0.024 ±0.004 a	0.028 ±0.004 a	0.023 ±0.004 ab

Note: * Same letters within the same lane mean non-significant difference (p >0.05).

Results of CAT and SOD determination are shown in Figure 4 and Figure 5, respectively. Treatment had a significant influence on both enzymes (p <0.05). Alloxan-induced diabetes caused a significant decrease in activities of both enzymes, while magnesium supplementation restored those values, causing even over-production of catalase. Both enzymes are important in detoxifying ROS produced by alloxan, with SOD reducing superoxide radicals to hydrogen peroxide and molecular oxygen, and catalase breaking down hydrogen peroxide into water and oxygen **(Lenzen, 2008)**. These reactions force Fenton reaction, and therefore, production of hydroxyl radical, the major damaging ROS related to β cell death, to halt **(Lenzen, 2008; Munday, 1988; Winterbourn and Munday, 1989)**. It was shown that excess of catalase production in liver hepatocytes, also possessing GLUT2 transporter and suitable for alloxan selective absorption, halted toxic effects of alloxan related to ROS production to the minimum **(Grankvist et al., 1979; Tiedge et al., 1997)**.

Data for G-SH and G-Per are shown in Table 3, with treatment having a significant influence on their values (p <0.05). The results for G-Red were non-significant (p >0.05, Table 3). Reduced glutathione is an important thiol-containing molecule, the presence of which causes alloxan- dialuronic acid cycling, resulting in the eventual production of ROS **(Lenzen, 2008)**. Alloxan-induced diabetes reduces levels of G-SH, and therefore, decreases reduced glutathione/oxidized glutathione ratio (G-SH/GSSG). However, increased levels of G-SH in β cells have a protective effect for glucokinase ("glucose sensor") inhibition by alloxan, as alloxan is kept in its oxidized form, dialuronic acid, incapable of reacting with thiol groups of glucokinase and deactivating it **(Ighodaro, Adeosun and Akinloye, 2017)**. Secondly, increased levels of G-SH cause activation of glutathione peroxidase, one of the enzymes capable of hydrogen peroxide deactivation. Progressed and established alloxan-induced diabetes reduced residual levels of G-SH in pancreatic tissue, though magnesium supplementation partially restored) G-SH levels to the value found in control animals (Table 3). A similar effect was observed with G-Per (Table 3), which also decomposes hydrogen peroxide. Glutathione reductase, which restores reduced glutathione levels through the reduction of its oxidized form, GSSG, using NADPH as reducing power, remained unchanged between treatments (Table 3).

Results for LDH and G6PD activity in pancreatic tissue after late established alloxan-induced diabetes are shown in Figure 6 and Figure 7. Treatment had a significant influence on both enzymes (p <0.05), with a significant decrease in their activities during late established diabetes (group D), and partial restoration in the case of magnesium supplementation after the onset of the disease in late progression. G6PD is a rate-limiting, first enzyme involved in the pentose phosphate pathway, which was shown to be inhibited during diabetes in the kidney cortex **(Xu, Osborne and Stanton, 2005)**. Pentose phosphate pathway generates extra reducing power in the form of NADPH, which can serve a substrate for glutathione reductase, which transforms oxidized GSSG into reduced G-SH, improving antioxidative "health" of the cells **(Xu, Osborne and Stanton, 2005)**. The LDH is the final enzyme in anaerobic oxidation of glucose, transforming pyruvate into lactate, and vice versa. Enzymatic activities of both LDH and G6PD in control untreated samples may serve as overall cell health state evaluation, and reduction in their activities may indicate impaired cellular health.

Several mechanisms may cause Mg^{2+} oral supplementation positive influence on pancreatic tissue, and specifically β cells, during experimentally induced diabetes. Magnesium complex with ATP activates glucokinase, an enzyme that serves as a "glucose sensor" as an initial step in cascade reactions of insulin release **(Gommers et al., 2016)**. Though it may happen even at a low sub-physiological level, some specific association between Mg-ATP and glucokinase may still exist **(Molnes et al., 2011)**, possibly decreasing alloxan inhibition of glucokinase. Intracellular magnesium concentrations inhibit L-type Ca^{2+} opening, avoiding massive Ca^{2+} influx inside cells, and "preserving" immediate insulin release and initial severe hypoglycemia caused by alloxan. Massive Ca^{2+} influx could also be one of the reasons for β cells death, besides ROS production **(Szkudelski, 2001)**. Magnesium serves as an antagonist of calcium, and more importantly, the ratio between Ca and Mg, not concentration levels themselves, causes insulin vesicle exocytosis **(Atwater et al., 1983)**. Additionally, magnesium dietary deficiency decreased antioxidant enzyme activities, namely, superoxide dismutase and catalase, in rat cardiac tissue, causing oxidative injury and explaining the pathogenesis of cardiac lesions in Mg-deficient rats **(Kumar and Shivakumar, 1997)**. A similar protective mechanism may be applied to magnesium influence on antioxidant enzymes in the pancreas. Contrary, although magnesium-deficient diet in rats caused decreased reduced glutathione levels in erythrocytes, implying its role in the maintenance of G-SH concentration to protect against oxidative damage in the erythrocyte membrane, the magnesium-deficient diet caused increased reduced glutathione levels in liver and kidneys, with no influence on other soft tissues **(Hsu, Rubenstein and Paleker, 1982)**. **Chaudhary, Boparai and Bansal (2007)** also showed an individual effect of low magnesium diet, as well as a combined effect of low magnesium with high sucrose diet on the development of oxidative stress in rats, namely, increase in plasma and liver lipid hydroperoxide levels, decrease in plasma reduced glutathione, decrease in liver superoxide dismutase, glutathione-S-transferase, and catalase enzyme activity.

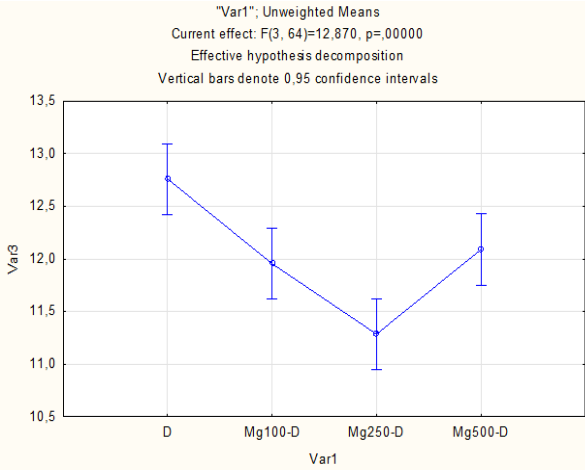

Figure 2 Influence of treatment (D, Mg100-D, Mg250-D, Mg500-D) on the mean value of fasting blood serum glucose levels on different days (day 21, 25, 27, 30). Error bars reflect standard error of mean. Adapted from Statistica ver 10.0.

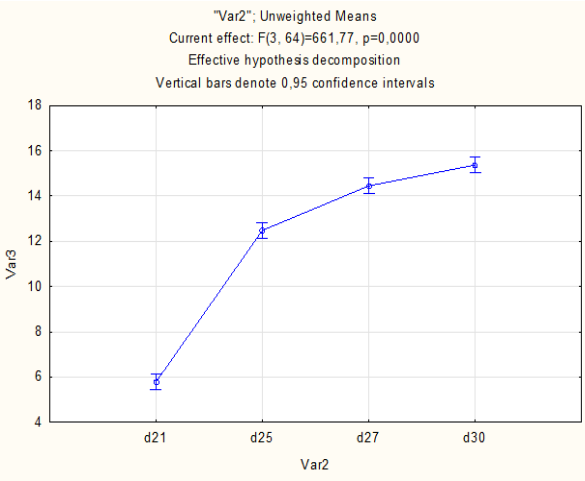

Figure 3 Influence of time factor (day 21, 25, 27, 30) on the mean value of fasting blood serum glucose levels in diabetic (D), magnesium supplemented diabetic rats (Mg100-D, Mg250-D, Mg500-D). Error bars reflect standard error of mean. Adapted from Statistica ver 10.0.

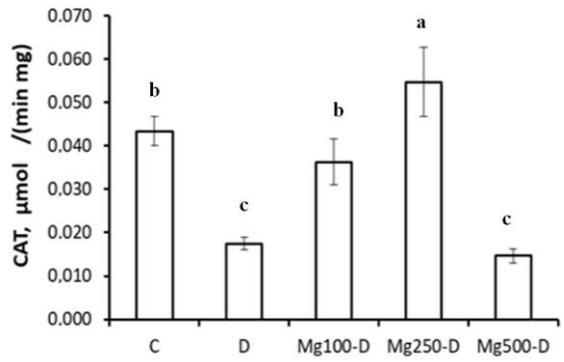

Figure 4 Mean values of pancreatic catalase (CAT) activity in control (C), diabetic (D), magnesium supplemented diabetic rats (Mg100-D, Mg250-D, Mg500-D) as measured on day 30 (sacrifice day). Error bars reflect standard deviations. Means with the same letters are not significantly different (p >0.05).

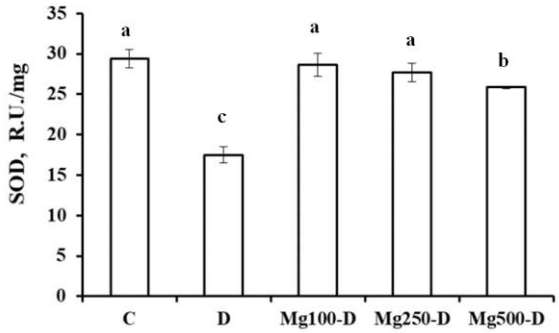

Figure 5 Mean values of pancreatic superoxide dismutase (SOD) activity in control (C), diabetic (D), magnesium supplemented diabetic rats (Mg100-D, Mg250-D, Mg500-D) as measured on day 30 (sacrifice day). Error bars reflect standard deviations. Means with the same letters are not significantly different (p >0.05).

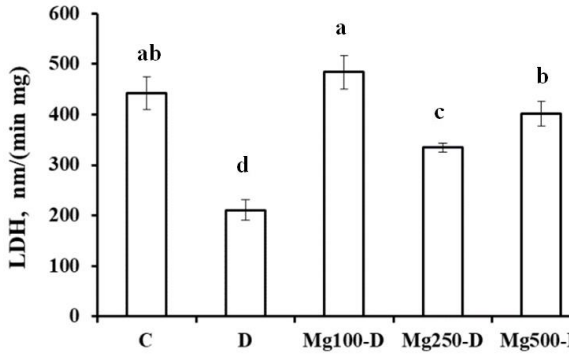

Figure 6 Mean values of pancreatic lactate dehydrogenase (LDH) activity in control (C), diabetic (D), magnesium supplemented diabetic rats (Mg100-D, Mg250-D, Mg500-D) as measured on day 30 (sacrifice day). Error bars reflect standard deviations. Means with the same letters are not significantly different (p >0.05).

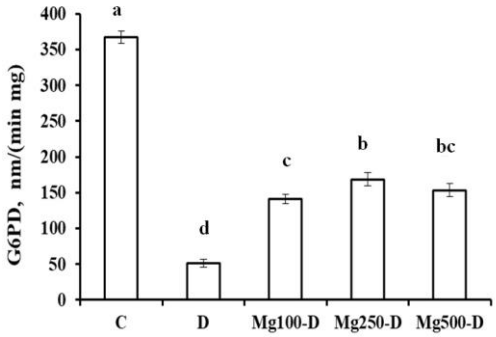

Figure 7 Mean values of pancreatic glucose-6-phosphate dehydrogenase (G6PD) activity in control (C), diabetic (D), magnesium supplemented diabetic rats (Mg-100D, Mg250-D, Mg500-D) as measured on day 30 (sacrifice day). Error bars reflect standard deviations. Means with the same letters are not significantly different (p >0.05).

CONCLUSION

To summarize, daily oral magnesium supplementation at a dose of 250 $mg.kg^{-1}$ live weight in advance before alloxan-induced diabetes and within 10 days of the diabetic state improved fasting glucose levels in rats, though did not prevent the onset of diabetes. Oral supplementation with magnesium restored glucose metabolism enzymes levels, such as LDH and G6PD, in diabetic rat pancreas, compared to alloxan-induced diabetes alone, which suppressed their activity. Additionally, antioxidant system enzymes, namely, catalase, superoxide dismutase, glutathione peroxidase, and reduced glutathione levels, all improved in pancreatic tissue after magnesium administration in late-stage diabetic rats. Inconclusive results were obtained regarding lipid hydroperoxide, either due to technical issues or lack of influence of magnesium on the pancreas due to very late testing after the development of diabetes.

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