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The influence of hypomagnesemia on erythrocyte antioxidant enzyme defence system in mice

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Abstract

The effect of magnesium deficiency on antioxidant defence system was studied in RBC of mice suffering from hypomagnesemia. The animals were kept for 8, 15 and 22 days on magnesium-deficient diet with consequent reduction of magnesium level in plasma by 38% at the first 8 days and by 64% after 22 days of experiment. The activities of the most important antioxidant enzymes, catalase, glutathione peroxidase, superoxide dismutase, glutathione reductase, glutahione S-transferase were assayed in hemolysates. The level of reduced glutathione in erythrocytes was measured as well. Apart from catalase, the activities of antioxidant enzymes were decreasing. The activity of superoxide dismutase decreased gradually during the experiment and on the 15th and 22nd day of experiment was significantly ($P < 0,05$) lowered by 30 and 32% respectively. The catalase activity was increased on each point of the experiment with the peak value up to 149% on 15th day, and by 32% on 22nd day. Glutathione peroxidase activity was insignificantly reduced. The reduction of Glutatione reductase and Glutathione S-transferase activities by 24 and 21%, respectively, were observed after 8 days of the experiment with a further downward tendency. The reduced glutathione was significantly depleted after 8 days by 33% and was kept on that level in the course of the study. These findings support previous reports on the hypomagnesemia – induced alteration in endogenous enzyme antioxidant defences and glutathione redox cycle of mice.

Abbreviations: CAT – catalase; GSH – reduced glutathione; GSSG – oxidized glutathione; GSSG-R – glutathione reductase; GST – glutathione S-transferase; GPX – glutathione peroxidase; HYPO 8, HYPO 15, HYPO 15, HYPO 22 – study groups received hypomagnesemic diet for 8, 15 and 22 days, respectively; RBC – red blood cell; ROS – reactive oxygen species; SOD – superoxide dismutase.

Introduction

Erythrocytes are biconcave discs averaging about 7.7 μM in diameter. The main function of RBC is to provide the tissue with oxygen and remove carbon dioxide and protons produced in metabolic processes. This important function must be performed efficiently, therefore the structure of RBC is completely subordinate to their tasks. The plasma membrane of the erythrocytes contains about 15 major proteins, is selectively permeable and encloses the solution of cytoplasmic proteins, $\sim$ 33% of which is haemoglobin. In mammals, the erythrocyte cytoplasm lacks a nucleus and such substrates as mitochondria, lysosomes and Golgi apparatus. ATP needed for metabolic functions derives from nonoxygen glycolysis.

The RBCs are continually exposed to ROS mainly through the release of O_2^- from oxyhaemoglobin and in part, through the Fenton reaction of haemoglobin, which generates the powerful oxidant hydroxyl radical (Giulivi *et al.* 1994). It has been estimated that about 3% of the haemoglobin undergoes oxidation every

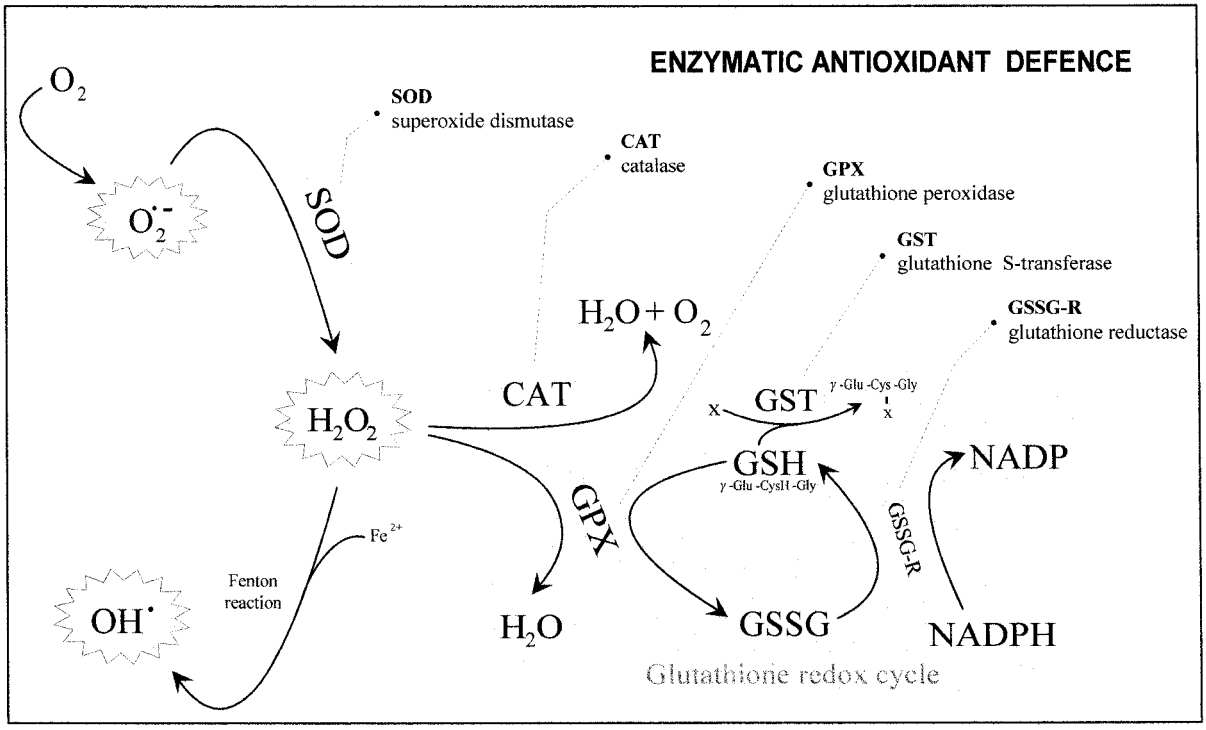

Figure 1. Enzymatic antioxidant enzymes in RBC.

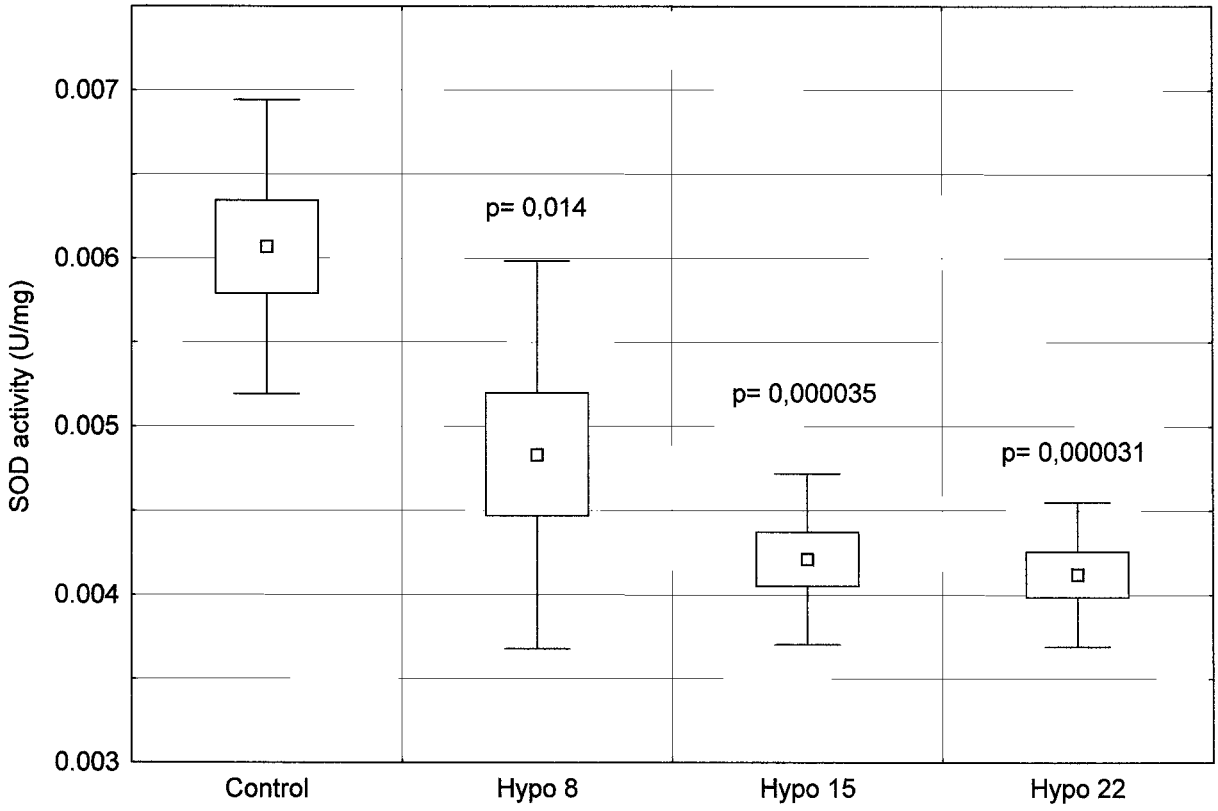

Figure 2. Activity of superoxide dismutase in the course of the experiment.

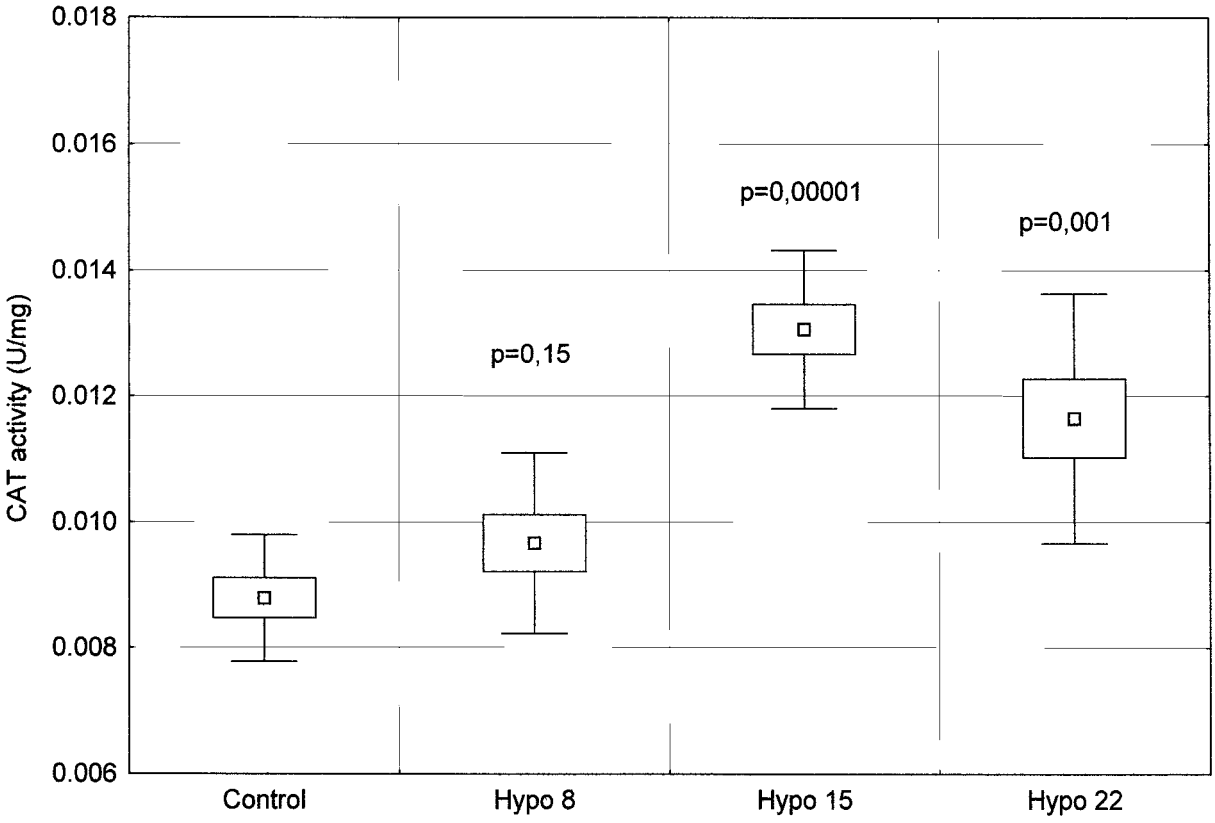

Figure 3. Activity of catalase in the course of experiment.

day, producing methaemoglobin. The haem ring coordinated with O_2 may, after its disintegration, release $O_2^{\cdot -}$ (Balagopalakrishna *et al.* 1996; Marden *et al.* 1995).

SOD rapidly converts O_2^- to H_2O_2, which is removed by catalase and GPX enzyme. Some amount of H_2O_2 may react with iron ion, derived from degradated haem protein. The resulted $OH^{\cdot}$ radical has the most harmful influence on RBC integrity. Polymorphonuclear cells, such as neutrophils and eosinophils, which release superoxide anion and other free radicals when activated, are another potential source of ROS in the surroundings of RBC (She *et al.* 1989; Wozniak *et al.* 1989).

Mg deficiency induces a systemic proinflammatory state, followed by the secretion of inflammatory cytokines (Weglicki *et al.* 1992b, 1994, 1996). As an outcome of such situation, activated macrophages and other cells produce ROS in excess (Dickens *et al.* 1992; Weglicki *et al.* 1996; Wiles *et al.* 1997).

ROS generated physiologically and pathologically from various intracellular and extracellular sources have harmful influence on their surroundings. Oxidative stress develops when the levels of antioxidants are lowered or when production of free radicals exceeds the capacity of the cell to dispose of them. As a result of ROS activity several irreversible modifications of biologically fundamental macromolecules have been described, including oxidation of purine nucleotides of nucleic acid, oxidation of protein -SH group and initiation of lipid peroxidation reaction chain (Halliwell *et al.* 1998). RBCs are particularly exposed to ROS, as they lack the ability to synthesise new proteins and lipids.

RBCs have evolved several antioxidant strategies aimed at the detoxification of ROS. One of them is enzymatic antioxidant defence system, consisting of agents that catalytically remove ROS. The examples are the enzymes SOD, CAT, GPX and GSSG-R (Gaetani *et al.* 1989; Eaton 1991; Kurata *et al.* 1993) (Figure 1).

Glutathione redox cycle is composed of the enzyme GPX and GSSG-R and the cosubstrates GSH and NADPH. Intracellular GSH and GSH-dependent

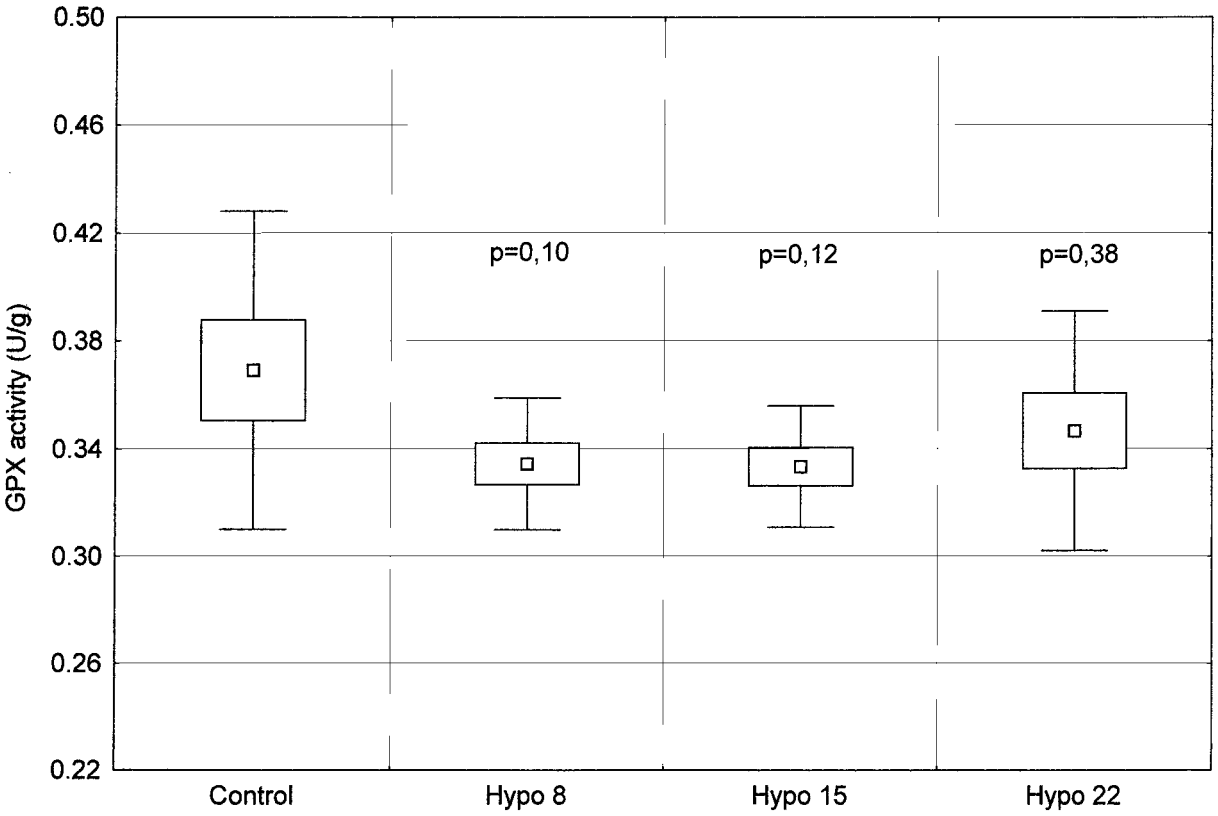

Figure 4. Activity of glutathione peroxidase in the course of experiment.

enzymes provide cellular protection against oxidant by using peroxidase to reduce hydrogen or lipid peroxide, with the concurrent oxidation of GSH to glutathione disulfide GSSG. GSSG is reduced back to GSH by GSSG-R through the reducing power of NADPH provided by the pentose phosphate pathway. In addition GSH is a cosubstrate for detoxification of some toxic agents by glutathione S-transferases and also may scavenge ROS including hydroxyl radical, singlet oxygen, nitric oxide and peroxinitrite.

The aim of the study was to investigate the enzyme antioxidant mechanisms in response to a proinflammatory state induced by magnesium deficiency.

Material and methods

In our experiments we used 9–11 week male C 57/bl mice. The animals were divided into 4 groups (10 animals per group): The control group received normomagnesemic diet (Altromin, Germany) and deionized water ad libitum, and 3 study groups received hypomagnesemic diet (Altromin, Germany) and deionized water ad libitum for 8, 15 and 22 days.

Animals were kept under standard laboratory condition with 12:12 h light:night cycle.

After the experiments animals were anaesthetised with ketamine and blood was taken by the cardiac punction, collected into heparinized tubes, and centrifuged for 10 min at 1,853 $\times$ *g* at 4 °C. The buffy coat was discarded, and erythrocytes were washed twice with large volumes of phosphate-buffered saline. Hemolysates were prepared by diluting RBC 1:4 with deionised water and stored at −75 °C for further assessment.

SOD activity was measured using spectrophotometric method (McCord & Fridovich 1969). In this method, the inhibition of the cytochrome c reduction

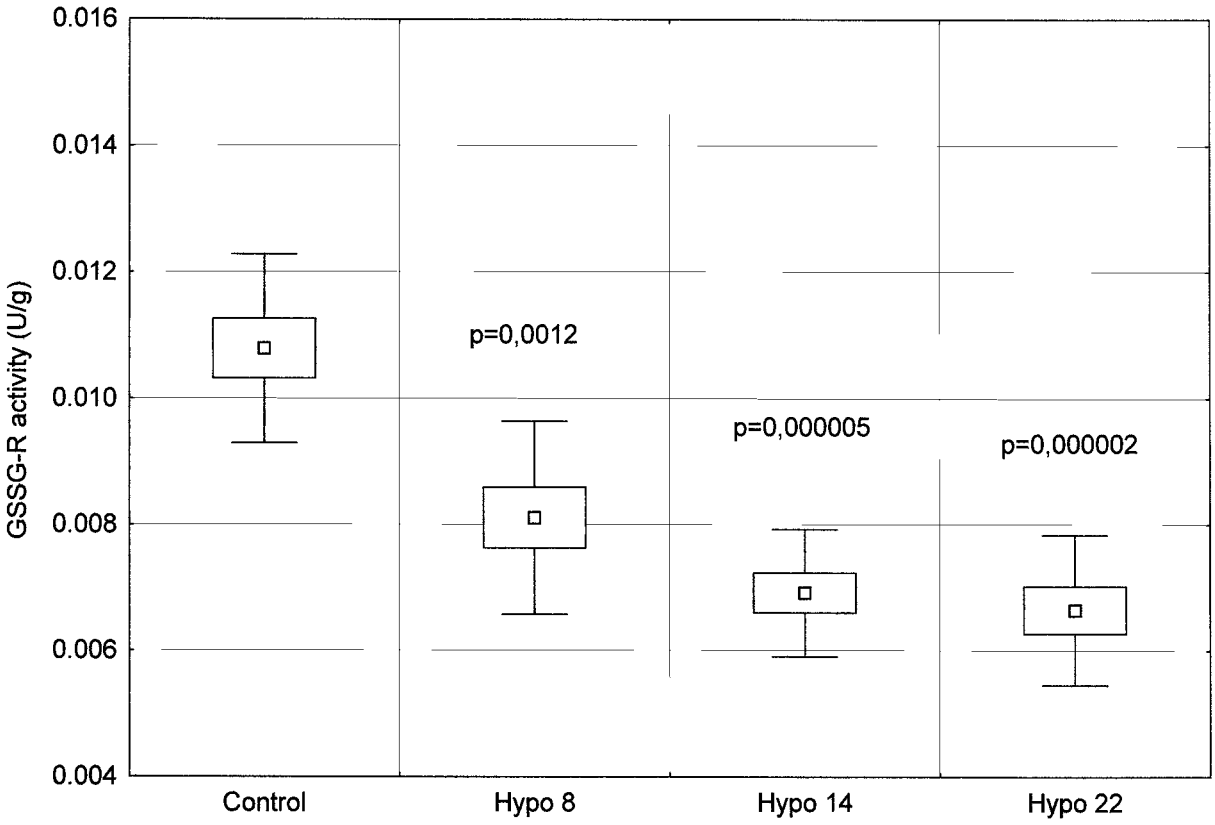

Figure 5. Activity of glutathione reductase in the course of experiment.

rate is monitored at 550 nm at 25 °C, utilising the xanthine/xanthine oxidase system as the source of $O_2^{\cdot-}$. SOD competes for superoxide and decreases the reduction rate of cytochrome c. One unit of SOD activity is defined as the amount of enzyme that inhibits by 50% the rate of cytochrome c reduction.

GPX activity was measured by following the rate of oxidation of the reduced form of glutathione, which was similar to the method previously reported (Paglia & Valentine1967). The formation of oxidised glutathione was monitored by a decrease in the concentration of NADPH, measured at 340 nm and 37 °C, caused by the addition of glutathione reductase to the reaction mixture.

CAT activity was measured using a method reported by Aebi 1984. This method uses the change in absorbance at 240 nm and 25 °C of a solution of 10 mM H_2O_2 in 50 mM phosphate buffer, pH 7.0. The decrease in absorbance per unit time is a measure of the catalase activity.

GST activity in the erythrocytes was measured by using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate according to the method described by Habig *et al.* 1974. The reaction mixture in 2 ml contained 0.2 M sodium phosphate buffer (pH 6.5), 0.95 mM CDNB, 0.95 mM GSH, and 0.5 ml lysed erythrocyte suspension. The reaction was initiated by the addition of the electrophilic substrate CDNB and the reaction was monitored at 25 °C spectrophotometrically by the increase of the absorbance at 340 nm.

GSSG-R activity was determined as described by Carlberg and Mannervik, by following NADPH oxidation at 340 nm. Hemolysates samples were incubated in the presence of 0.5 mM GSSG and 0.15 mM NADPH, in 0.1 M sodium phosphate buffer, pH 7.0. One unit of GSSG-R was defined as the amount of enzyme that catalyzes the oxidation of one micromole of NADPH per minute (Carlberg & Mannervik 1995).

Glutathione level was measured fluorimetrically with use of o-phthalaldehyde as a fluorescent reagent (Hissin & Hilf 1976).

The level of magnesium in plasma and RBCs was assessed by atomic absorption spectrophotometry.

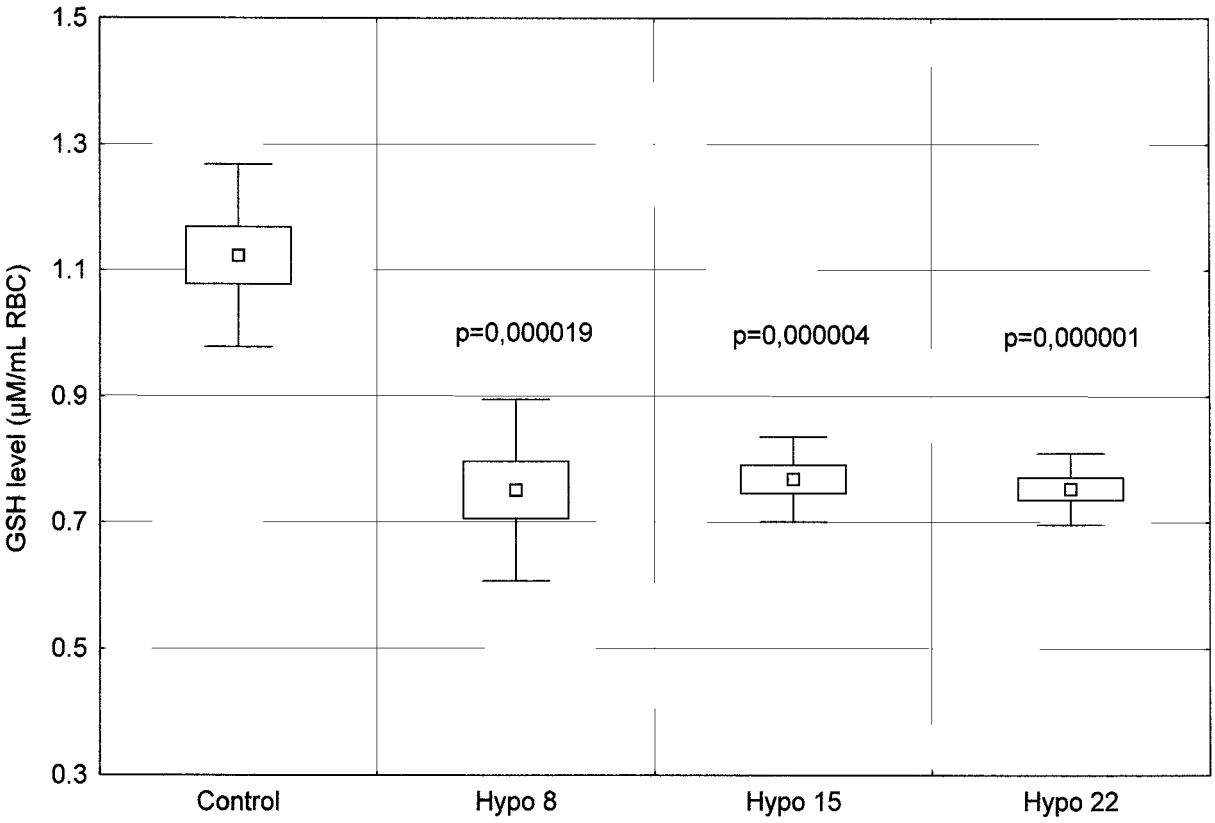

Figure 6. The level of glutathione in the course of experiment.

Protein concentration was assessed by Lowry method (Lowry *et al.* 1951).

All enzyme activities were reported as unit per mg or g of protein in hemolysates.

Statistical analyses

Data are expressed as the means ± standard error and ± standard deviation. Differences between groups were analysed by Student's *t*-test. A P-value <0.05 was considered to be significant.

Results

Magnesium level in plasma of these C 57/bl mice, which received diet with a trace amount of magnesium decreased systematically, and was reduced by 38%, 44% and 64% after 8, 15 and 22 day of experiment, respectively. Despite the large continuous decrease in plasma magnesium level, the loss of magnesium from RBCs was assessed to be maximum 23% on the 15th day of the experiment (data not shown).

The activity of SOD decreased gradually in a time of the study (Figure 2). Significant reduction by 20% was observed already after 8 days, after 15 and 22 day the activity was reduced by approximately 32%.

CAT activity increased gradually. After 8 days a mild increase and after 15 and 22 day significant increase by 49 and 32% has been measured (Figure 3).

The reduction of GPX activity has been estimated to be insignificant (Figure 4). The magnesium depletion resulted in a decrease of GSSG-R and GST in a time of experiment. After 22 day the loss of activities of both enzymes was closed to 35% (Figures 5 and 7). The RBC capacity of glutathione was depleted by 33% after 8 day and that lowered level was maintained to the end of experiment (Figure 6).

Discussion

Magnesium plays an important role in physiology of mammalians metabolism, taking part in many biochemical processes, as a component or activator of

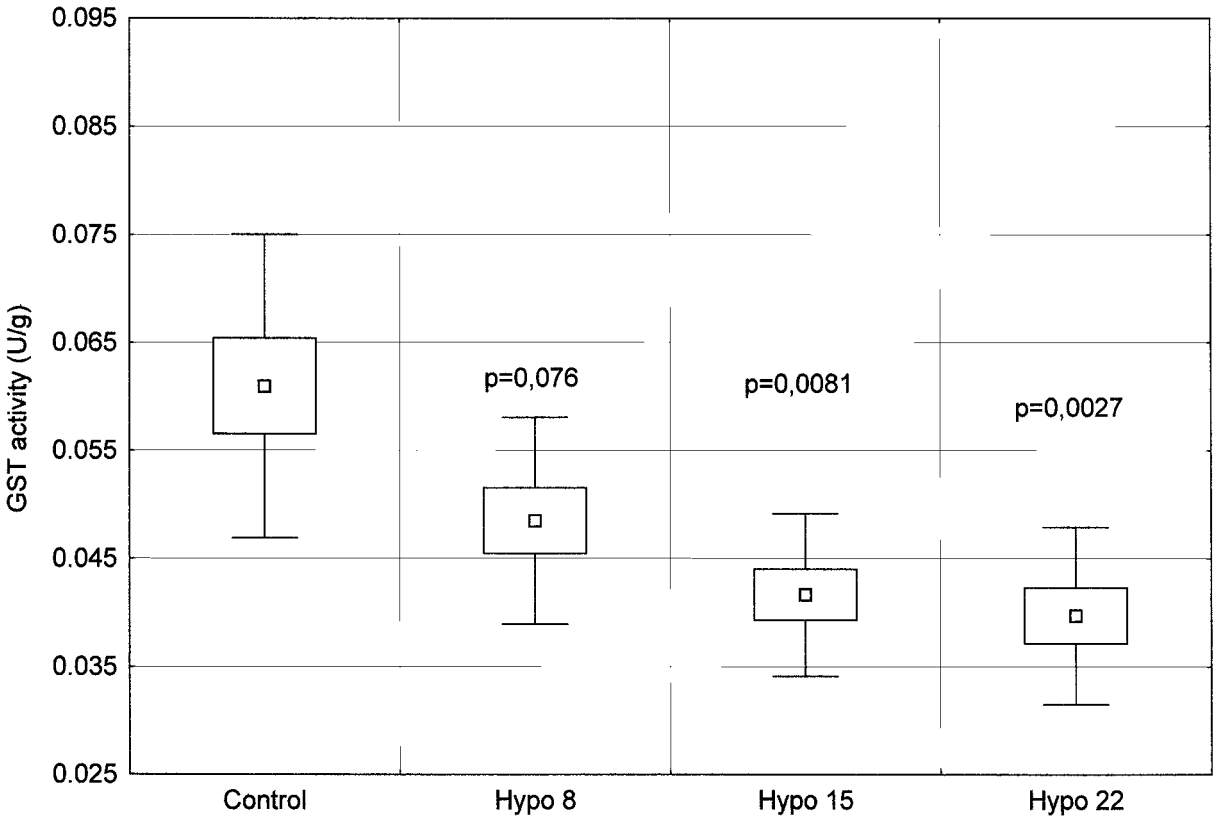

Figure 7. Activity of glutathione S-transferase in the course of experiment.

enzymes involved in carbohydrates, lipid and protein metabolism (Ryan 1991).

In human and in animal models, Mg deficiency leads to severe biochemical dysfunction and induces a variety of pathologies as a consequence of possible oxidative damage caused by free radicals (Astier *et al.* 1996; Robeson *et al.* 1980; Bloom 1988; Rude 1993; Whang *et al.* 1994). Oxidative stress, possibly induced by Mg-deficiency, is defined as a disturbance between the prooxidant and antioxidant balance in favour of the former. Both situations may contribute to pathologies observed in magnesium deficiency.

Dietary restriction of Mg induces changes in circulating inflammatory cytokines (IL 1, 6, TNF-α), that activate a number of cells (macrophages, neutrofils and endothelial cells) to generate oxyradicals (Weglicki & Phillips 1992a). In Mg-deficiency, release and excretion of catecholamines were increased (Caddell *et al.*1986). These biologically-important molecules oxidize in the presence of oxygen to yield O_2^-. Hence RBC of hypomagnesemic animals are constantly subjected to an increased prooxidant environment. Antioxidant defence also seems to be altered during magnesium deficiency. Animals subjected to hypomagnesemia had decreased level of glutathione in blood and changed activity of antioxidant enzymes in hart and liver (Zhu *et al.* 1993; Mak *et al.* 1994; Kumar & Shivakumar 1997; Kuzniar *et al.* 2001). However, the effect of Mg deficiency on RBC antioxidant enzyme has not been fully studied yet.

Oxidative stress can produce cell injury by multiple pathways. Proteins and lipids damaged by oxygen radicals or other ROS can no longer normally function. Lipid peroxidation and oxidation of membrane protein result in erythrocyte membrane damage. As erythrocytes can not synthesise damaged molecule de novo, repeated damages accelerate their death. It has been shown that erythrocytes from magnesium-deficient hamster display an enhanced susceptibility to oxidative stress (Freedman *et al.* 1992).

Different antioxidants systems are needed to protect cells against harmful action of ROS. In our experiment the influence of hypomagnesemia on activities of antioxidant enzymes and glutathione levels has been studied.

In the processes of autooxidation of haemoglobin a certain amount of O_2^- comes into existence. In physiological condition it is removed as a result of SOD activities. The results of our experiment indicate that activity of SOD significantly decreased upon exposure to magnesium deficient diet and further decreased in the course of experiment. Apart from SOD, RBSs have no other mechanism to remove constantly arising O_2^-. As SOD can not efficiently withdraw O_2^-, erythrocytes with low SOD activities are exposed to its harmful effect. Hydrogen peroxide rises in the cells from O_2^-, as a result of SOD activity, and partly in activated neutrophils and macrophages by myeloperoxidases activity. Overproduction of H_2O_2 by stimulated neutrophils and macrophages occurs during experimental hypomagnesemia, increasing in this way the amount of H_2O_2 in RBC surroundings. CAT and GPX are responsible for enzymatic removal of H_2O_2. An increase of CAT activity, observed during the experiment, may be a consequence of adaptation changes in response to increased H_2O_2 production. Similar tendencies regarding CAT and SOD activities were observed in our previous experiment in heart tissue (Kuzniar *et al.* 2001).

The ability of glutathione redox cycle seems to be inefficient in detoxifying ROS, as the activity of enzymes it consists of, decreases in a course of experiments. Activity of GPX slightly decreases. The activities of remaining enzymes GSSG-R and GST significantly decrease.

The pool of GSH, a cofactor of GPX was determined to be decreased. That fact can be associated with mild decrease of GPX activity also observed in that experiment. The reduction of GSSG by GR and processes of biosynthesis from aminoacids maintain GSH on the adequate level, making it available for GPX and GT. The decreased level of GSH may be a consequence of GSH consumption by GPX and GST or reduced synthesis and transformation by GSSG-R. There may also exist an efflux of GSH from RBC by destroyed membranes.

The glutahione level and activities of antioxidant enzymes excluding CAT have been shown to be depleted, which indicates that RBC have depleted antioxidant mechanisms in a response to magnesium deficiency. In the case of overproduction of H_2O_2 and inefficient mechanism of its removal, the most reactive radical $OH^{\cdot}$ may arise from H_2O_2, by a Fenton reaction within RBC.

At the current scientific knowledge and the stage of presented research it is impossible to explain the mechanism of the reported changes. There are several possible points of interaction between Mg and antioxidant enzymes. As magnesium ion is required in processes of transcription and replication of studied enzymes, its deficiency may affect antioxidant system of RBC as early as in erythropoietic processes. Because the half-life of RBC is 15 to 25 days a relatively big number of erythrocytes is replaced during the experiments (Benirschke *et al.* 1978). The increased level of peroxidised products observed during hypomagnesemia may induce structural and functional alteration of examined enzyme, thus the deficiency of Mg may also influence the activity of examined enzymes.

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