

IMPORTANCE OF MAGNESIUM SUPPLEMENTATION IN CHILDREN WITH ATOPIC BRONCHIAL ASTHMA

Summary of Ph.D. thesis

Olga Bede M.D.

Faculty of Medicine of the University of Szeged, Hungary
Paediatric Department

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ABBREVIATIONS

APH	acetylphenylhydrazine
Br	bilirubin reductase
DTNB	5,5'-dithio-bis-2-nitrobenzoic acid
FEV1	forced expiratory volume in 1 second
GINA	Global Initiative for Asthma
Gr	glutathione reductase
GSH-Px/Gpx	glutathione-peroxidase
GSH	reduced glutathione
GSSG	oxidized glutathione
G6PD	glucose-6-phosphate dehydrogenase
Hb	haemoglobin
HMP	hexose monophosphate
H₂O₂	hydrogen peroxide
Ho	haemoxigenase
metHb	methaemoglobin
Mg	magnesium
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NEM	<i>N</i> -ethylmaleimide
O₂^{·-}	superoxide
·OH	hydroxyl radical
oxyHb	oxyhaemoglobin
RBC	red blood cell
ROS	reactive oxygen species
SOD	superoxide dysmutase

INTRODUCTION

Bronchial asthma is characterized by variable and reversible airflow obstruction and by bronchial hyperresponsiveness in response to variety of apparently unrelated stimuli. Several inflammatory cells are found in asthmatic airways, including mast cells, macrophages, eosinophils, basophils, neutrophils, lymphocytes and platelets. These cells release a variety of mediators which interact in a complex manner to produce the pathophysiological features of asthma.

Bronchial asthma is associated with uncompensated oxidative stress, which accompanies chronic airway inflammation and cell membrane and receptor damage.

Several of the inflammatory cells such as mast cells, macrophages, eosinophils, neutrophils release reactive oxygen species (ROS) after activation by a variety of stimuli. They generate superoxide ($O_2^{\cdot-}$) which is rapidly converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). The hydroxyl radical ($\cdot OH$) is formed nonenzymatically in the presence of Fe^{2+} as a secondary reaction. In neutrophils myeloperoxidase also results in the formation hydrochlorous acid (HOCl) from H_2O_2 in the presence of chloride ions. HOCl is a potent oxidant. Several stimuli may release $O_2^{\cdot-}$ from eosinophils, including opsonized zymosan, complement fragments, IgG and IgE. In the presence of halide ions, the enzyme eosinophil peroxidase and H_2O_2 form a potent cytotoxic system against a variety of cells. Alveolar macrophages also generate oxygen metabolites by several stimuli, including IgE. Oxygen metabolites which are generated by inflammatory cells may stimulate nearby cells to release oxygen metabolites in a self-perpetuating cascade. Additionally, the airway epithelial cells themselves produce ROS, which may also stimulate inflammatory cells to release reactive oxygen metabolites.

Oxidative stress develops when the levels of antioxidants are lowered and when production of free radicals exceeds the capacity of the cell to dispose of them. RBCs are particularly exposed to ROS as they lack the ability to synthesise new proteins and lipids. There are several antioxidant defence mechanisms which protect against ROS including the enzymes, SOD, catalase, glutathione peroxidase (GSH-Px). The main mechanism for generating reducing capacity in the erythrocytes is the pentose phosphate pathway. In this shunt off the main glycolytic pathway, glucose-6-phosphate is oxidized by nicotinamide adenine dinucleotide phosphate (NADP), in a reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PD), resulting in the production of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The activity of hexokinase catalysing the formation of glucose-6-phosphate is a Mg-

dependent process. NADPH enters the glutathione-dependent pathway by reducing oxidized glutathione (GSSG) to reduced glutathione (GSH) in a reaction catalyzed by glutathione reductase. A decrease in erythrocytes NADP biosynthesis leads to a diminution in cellular ability to reduce GSSG to GSH causing in a diminished ability of cells to defend against oxidant stress.

In human and 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. One mechanism by which Mg deprivation may increase cellular vulnerability to oxidation is by depleting reduced glutathione. In an oxidative environment there is an increased GSH consumption. The two enzymes that catalyze GSH synthesis, glutamate cysteine ligase (formerly called γ -glutamyl-cysteine synthetase) and glutathione synthetase, are both Mg-dependent.

The ratio of Mg retention to urinary excretion showed a significant inverse correlation with Mg concentration in erythrocytes. It has been shown that erythrocyte from Mg deficient hamster displayed an enhanced susceptibility to oxidative stress.

AIMS

First aim was to establish whether a Mg deficiency is indicated by a decreased urinary excretion and to determine whether 12-week oral Mg supplementation affects the Mg status and bronchodilator use in children with stable bronchial asthma in a randomized, double-blind, placebo-controlled study.

Second aim was to investigate the effect of long-lasting oral Mg supplementation on the RBC redox system in stable, persistently moderately asthmatic children in a randomized, double-blind, placebo-controlled study.

MATERIALS AND METHODS

SUBJECTS AND STUDY DESIGN

The first study was made of the effects of long-lasting oral Mg supplementation *on the serum total and free Mg levels and the Mg excretion in the urine*. 89 atopic asthmatic children (62 boys, 27 girls) aged 4-16 years were recruited. The patients exhibited persistent mild or moderate bronchial asthma on the GINA classification.

The same protocol was used to study the effects of long-lasting oral Mg supplementation *on the RBC redox system*. Forty atopic asthmatic children (28 boys,

12 girls) aged 4-16 years were recruited. The recruited patients for this study exhibited persistent moderate bronchial asthma on the GINA classification.

The diagnosis of bronchial asthma was based on the clinical, lung function, skin prick test, an elevated serum total IgE level, bronchial hyperreactivity and broncholysis test. Subjects were included only if their disease had been known for ≥ 6 months, their diet was left unchanged, Mg supplementation had not been administered in the 4-week run-in period, short-term inhaled beta2-mimetics had been used as rescue medication only in cases of dyspnoea and wheeze, inhaled or systemic corticosteroids and other asthma medication had not been administered and they had had a serum Mg level ≤ 0.83 mmol/l. The investigation ran over three 4-week treatment periods. During an initial 4-week period and the treatment period, the children ate their normal diet and short-term inhaled beta2-mimetics were administered if necessary. At each visit they received placebo or Mg capsules according to a randomized, double-blind protocol for the next 4-week period. The Mg intake was predetermined according to age within the groups. Children ≤ 7 years old received 200 mg, and children >7 years old received 290 mg Mg citrate daily, or 260 mg glucose as a placebo, in capsule form each evening. The number of bronchodilator doses and symptom scores each day were recorded on a personal diary card.

This study was approved by the Local Human Investigation Review Board of Albert Szent-Györgyi Medical University.

DETERMINATION OF MAGNESIUM

Mg forms a coloured complex with xylydyl blue in strongly basic solution, where calcium interference is eliminated by glycol ether diamine-N,N,N'-tetraacetic acid. The colour produced, measured biochromatically at 520/800 nm (Hitachi 917 equipment), is proportional to the Mg concentration. Free Mg concentration in blood after haemolysis and from 24-h urine samples was determined with a blood gas analyser (NOVA 8 equipment), using a Mg-selective electrode. Most current blood analysers require a whole blood sample of <100 μ l and measure pH, pCO₂ and pO₂ simultaneously.

DETERMINATIONS OF REDUCED AND OXIDIZED GLUTATHIONE

The concentrations of GSSG+GSH and GSSG in whole blood haemolysates in the presence of cold 0.01 M sodium phosphate buffer containing 5 mM EDTA (pH=7.5) were measured by previously accepted highly sensitive and specific standard

methods. Depending on the concentrations of GSH ($\mu\text{mol/g Hb}$) and GSSG (nmol/g Hb) during the first 6 min, samples for both GSSG and GSH were measured spectrophotometrically at 412 nm in the presence of DTNB (0.6 μmol), glutathione reductase (10 μg), and NADPH (0.2 μmol). Samples for GSSG were measured after the alkylation of GSH with NEM and after the separation of GSSG and the NEM-GSH complex by gel filtration with Sephadex G-10. The concentrations of GSH and GSSG were expressed with reference to Hb determined by the cyanmethaemoglobin method.

REDUCED GLUTATHIONE STABILITY TEST

Whole blood was incubated with 0.33 mmol APH and sufficient glucose at 37 °C for 60 min. The residual GSH was expressed as a percentage of the original concentration.

DETERMINATIONS OF OXIDIZED COMPONENTS OF HAEMOGLOBIN FROM HAEMOLYSATES

Changes in oxidized components of Hb such as oxyhaemoglobin, methaemoglobin and hemichrome from haemolysates were examined before and after *in vitro* incubation with APH. Values were expressed as percentages.

DETERMINATIONS OF PLASMA HAEMOGLOBIN, BILIRUBIN, OXYHAEMOGLOBIN, METHAEMOGLOBIN AND HEMICHROME

Heparinized plasma samples were diluted 1:40 (v/v) with 5 mmol PBS (pH=7.4) and measured spectrophotometrically at different absorption wavelengths.

RESULTS

MAGNESIUM STATUS IN MILD AND MODERATE ASTHMATICS

There was a significant increase in serum total Mg at the end of the study in both the Mg and the placebo treated patients in both the mild and the moderate asthmatics. The mean serum total Mg was significantly higher at the end in the magnesium group than in the placebo group in mild asthmatics, but not in moderate asthmatics.

A statistically significant decrease was found in serum free Mg at the end of the Mg treatment period in mild asthmatics without clinical consequences. In moderate asthmatics no significant change has been observed. There was no change in serum free Mg at the end of the placebo treatment period either in mild or moderate asthmatic patients.

The 24-hr Mg excretion was not significantly increased at the end of either the Mg or the placebo treatment period in mild asthmatics. There was a significant increase in

the mean 24-hr urine Mg excretion at the end of the Mg treatment and a significant decrease at the end of the placebo treatment in moderate asthmatics.

CHANGES IN FEV1 IN MILD AND MODERATE ASTHMATICS

There was a significant increase in FEV1 at the end of the study in both the Mg-treated and the placebo-treated patients either in mild or moderate asthmatic patients. A significantly more frequent use of short-term inhaled beta2-mimetics as rescue medication produced the same increase in FEV1 in the later phase of the study in both groups of placebo-treated patients.

BRONCHODILATOR USE

The frequency of bronchodilator use decreased significantly at the end of the study in the Mg-treated patients than in the placebo-treated patients with moderate asthma.

CHANGES IN SYMPTOM SCORES

A significant decrease in daytime symptoms was experienced at 8 weeks in the Mg-treated patients with moderate asthma. This decrease persisted at the end of the study. A significant change was also observed at 12 weeks in the placebo-treated patients with moderate asthma. No significant differences in any symptom scores were observed between the Mg-treated and the placebo-treated children with mild asthma.

CHANGES IN REDOX SYSTEM

Reduced and oxidized glutathione in the blood

There was a significant increase in GSH concentration in the Mg-treated patients after the 12-week treatment period, but not in the placebo-treated group. The GSSG concentration did not show any significant alterations during the 12-week treatment period in either the Mg-treated or the placebo-treated patients.

Glutathione redox ratio

A significant decrease in the molar ratio GSSG/GSH was observed in the placebo-treated patients after 12 weeks, but not in the Mg-treated patients. A significant decrease in GSSG/GSH associated with an insignificant decrease in GSSG concentration and a poorly increased GSH concentration, was found in the placebo-treated asthmatics.

Reduced glutathione stability test

The GSH stability test revealed that the residual capacity of the RBCs after an *in vitro* challenge with APH was significantly lower. The stability tests were approximately 42% before the treatment period and exhibited significant decrease at the end of the study in both the Mg-treated and the placebo-treated groups, as a sign of the increased sensitivity and the decreased antioxidant capacity of patients to resist an oxidative challenge.

Oxidized haemoglobin derivatives in the plasma

The plasma oxyHb concentration measured at 560 nm was not changed either in the Mg-treated or in the placebo-treated children after the treatment period as compared with the baseline values. However, the metHb, the hemichrome and the bilirubin values were significantly decreased to similar extents in both groups.

Whole blood and plasma haemoglobin concentrations

The plasma Hb levels were significantly decreased to different extents in both the Mg-treated and the placebo-treated groups. In parallel, we observed significant increases in the whole blood Hb concentrations in both groups.

Whole blood oxyhaemoglobin, methaemoglobin and hemichrome concentrations

Measurements of the whole blood oxyHb, metHb and hemichrome concentrations in the hemolysate were performed after the GSH determination. No significant differences before and after the treatment period were found in either group.

However, the oxyHb concentrations displayed significant decreases after an *in vitro* oxidative challenge with APH in both groups. The metHb concentration was considerably higher in the Mg-treated patients after acute oxidative stress than in the placebo-treated patients. There was no significant alteration in the hemichrome concentrations before and after the treatment period in either group (except for the hemichrome level which displayed an increasing tendency in the placebo-treated patients).

Linear regression between plasma haemoglobin concentration and plasma methaemoglobin and hemichrom levels

Analysis of regression demonstrated positive correlations, with significantly decreased plasma metHb and hemichrome levels and a decreased plasma Hb concentration in the Mg-treated patients at the end of the study.

CONCLUSIONS

1. Our study demonstrated that the total and free Mg level in the serum and urine in mildly or moderately asthmatic children on a normal diet were close to the lower levels of the normal range.
2. The measurement of the total and/or free Mg levels in the serum alone is not sufficient for a characterization of the Mg status.
3. Determination of the 24-hr urine Mg excretion is an important and simple way to assess a Mg deficit.
4. Based on the Mg excretion in urine, a Mg deficiency could be detected in moderately asthmatic children, who administered only short-term inhaled beta2-mimetics if necessary.
5. A significantly more frequent use of short-term inhaled beta2-mimetics as rescue medication produced the same increase in FEV1 in the later phase of the study in both groups of placebo-treated patients.
6. The significant reduction in bronchodilator use in children with moderate asthma versus placebo group suggests the benefit of Mg supplementation.
7. Improvement of the daytime clinical symptoms could be considered moderate during a 12-week Mg treatment period.
8. A long-term Mg supplementation was able to elevate the GSH concentration indicating that other compensatory mechanisms serving the defence against oxidative agents were activated to maintain the normal GSH level in children with moderate asthma.
9. The GSH capacity of the RBCs was about 42% in children with moderate asthma.
10. More significantly reduced plasma Hb concentrations and in parallel a significantly decreased plasma methHb and hemichrome levels which are products of the oxidative haemolysis were observed suggesting the benefit of Mg supplementation in the Mg-treated patients.

In general, nutritional Mg therapy for bronchial asthma palliates the coexistent primary Mg deficiency and its consequences.

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In extenso publications related to the Ph.D. thesis

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