IMPORTANCE OF MAGNESIUM SUPPLEMENTATION IN CHILDREN WITH ATOPIC BRONCHIAL ASTHMA

Ph.D. thesis

Olga Bede M.D.

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

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LIST OF PUBLICATIONS RELATED TO THE Ph.D. THESIS

I. Urinary magnesium excretion in asthmatic children receiving magnesium supplementation: a randomized, placebo-controlled, double-blind study.
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II. Effects of magnesium supplementation on the glutathione redox system in atopic asthmatic children
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III. Efficacy of magnesium in children with bronchial asthma
Olga Bede
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IV. A magnézium szupplementáció hatása az enyhe és közepesen súlyos asztmás gyermekek állapotára
Bede Olga, Surányi Andrea, Pintér Katalin, Szlávik Mária, Gyurkovits Kálmán
Gyermekgyógyászat, 53(2): 231-240. 2002.

V. A magnézium fiziológiája és patológiája
Bede Olga
Gyermekgyógyászat, 53(5): 587-593. 2002.

VI. Miért jó a magnézium asztmában
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VII. Az IgE szerkezete és funkciója
Bede Olga
Gyermekgyógyászat, 52 (2), 169-178. 2001.

VIII. Th2 citokintermelés
Bede Olga
Gyermekgyógyászat, 4, 406-12. 2001.

CONTENTS

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
H_2O_2	hydrogen peroxide
Ho	haemoxigenase
metHb	methaemoglobin
Mg	magnesium
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NEM	*N*-ethylmaleimide
$O_2^{\cdot -}$	superoxide
$^{\cdot}OH$	hydroxyl radical
oxyHb	oxyhaemoglobin
RBC	red blood cell
ROS	reactive oxygen species
SOD	superoxide dysmutase

1. INTRODUCTION

1.1. *Asthma as an inflammatory disease*

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 [Barnes *et al*. 1988, Foster *et al*. 2002, Schaerli *et al*. 2004, Rydell-Törmänen *et al.* 2006, Fixman *et al.* 2007].

1.2. *Oxidative stress in bronchial asthma*

Bronchial asthma is associated with uncompensated oxidative stress, which accompanies chronic airway inflammation and cell membrane and receptor damage [Doelman *et al.* 1990].

1.2.1. *Source of reactive oxygen species in bronchial asthma*

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 ($O_2^{\bullet -}$) which is rapidly converted to H_2O_2 by superoxide dysmutase (SOD). The hydroxyl radical ($^{\bullet}OH$) is formed nonenzymatically in the presence of Fe^{2+} as a secondary reaction [Halliwell *et al.* 1992]. 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 $O_2^{\bullet -}$ from eosinophils, including opsonized zymosan, complement fragments, IgG and IgE [Degenhart *et al*. 1986, She *et al.* 1989, Wozniak *et al*. 1989]. In the presence of halide ions, the enzyme eosinophil peroxidase and H_2O_2 form a potent cytoxic system against a variety of cells [Jong *et al*. 1980]. Alveolar macrophages also generate oxygen metabolites by several stimuli, including IgE [Kelly *et al*. 1988]. 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 [Lopez *et al.* 1988].

1.2.2. *Inhaled oxidants*

Reactive oxygen species may also be delivered to the airways by inhalation. Exogenous oxidants may be important in exacerbation of airway inflammation. Inhaled ozone and nitrogen dioxide may lead to an increase in airway responsiveness. It has been linked neutrophil infiltration in the airway epithelium. Ozone may inhibit the enzyme neutral endopeptidase in the airway epithelium that normally degrades peptides such as tachykinins, bradykinin and substance P. Cigarette smoke contains oxidizing free radicals, both in the gas phase and in tar [O'Byrne *et al*. 1984, Dusser *et al.* 1989, Martinez *et al*. 1988].

1.2.3. *Effect of reactive oxygen species on airway function*

Oxygen radicals are highly reactive close to cell membranes and may oxidize membrane phospholipids and may lead to cell death. They appear to oxidize certain amino acids in proteins such as methionine and cysteine and alter the function of proteins. $^{\bullet}OH$ can react with purine and pyrimidine bases of DNA, thiol compounds and the fatty acid side-chains of membrane phospholipids such as arachidonic acid and receptors on the cell surface. ß-adrenoreceptors containing sulfhydryl groups are sensitive to radicals [Risberg *et al.* 1991, Crastes de Paulet *et al.* 1987]. ROS, via lipid peroxidation, may provoke the release of arachidonic acid from membrane phospholipids and may lead to produce prostaglandins and leukotrienes [Taylor *et al.* 1983].

1.2.3.1. *Effect on airway epithelium*

Epithelial shedding is a characteristic feature of asthmatic airways. ROS generated during the inflammatory response might contribute to this shedding [Laitinen *et al*. 1985, Baesley *et al.* 1989]. When guinea pig tracheal epithelium is exposed to H_2O_2, there is increased transit of labelled terbutaline across the epithelium suggesting that there may be separation of the epithelial cells, allowing more rapid transit of certain molecules. This correlates with histological changes [Jeppsson *et al.* 1990].

1.2.3.2 *Effect on airway secretion*

Reactive oxygen species stimulate the release of high molecular weight glycoconjugates from the airway epithelial cells. This increased mucus secretion is depend on cyclooxygenase products. Exogenous oxidants and endogenous ROS from airway inflammatory cells might contribute to the mucus hypersecretion in asthma [Adler *et al.* 1990].

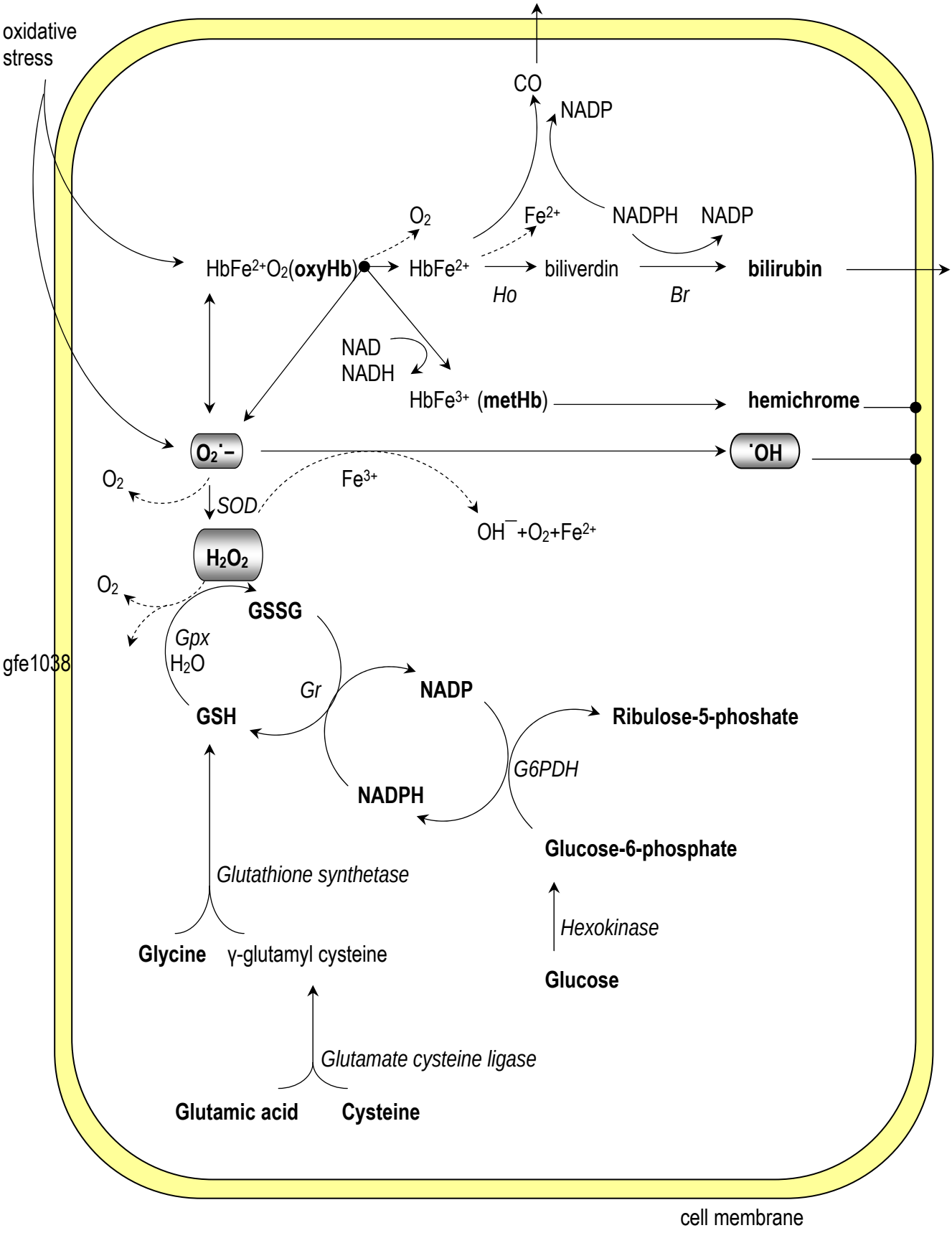
1.2.3.3 *Effect on vessels*

Oxygen radicals may lead to microvascular leak and oedema in airways. They cause increased vascular permeability via a direct damaging of vascular endothelial cells or via a release of eicosanoids [Tate *et al*. 1984, del Maestro *et al*. 1981].

1.3. *Role of antioxidants in bronchial asthma*

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 synthesize new proteins and lipids [Halliwell *et al.* 1998]. There are several antioxidant defence mechanisms including the enzymes, SOD, catalase, glutathione peroxidase (GSH-Px) which protect against ROS. The concentration of latter enzyme is lower in asthmatics. It requires selenium as a cofactor and has a selenocysteine residue at its active site. In asthmatic patients a reduction in both whole blood and plasma selenium and GSH-Px concentrations was observed [Flatt *et al.* 1990, Beasley *et al.* 1991, Hasselmark *et al.* 1993]. 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 NADP, in a reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PD), resulting in the production of 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 GSSG to GSH in a reaction catalyzed by glutathione reductase [Paglia 1995, Figure 1]. 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.

FIGURE 1. Effects of oxidative stress on heme metabolism and activation of glutathione redox system



1.4. *Relation between magnesium and oxidative stress*

Magnesium (Mg) is the second most abundant intracellular cation and is involved in numerous physiological functions, including protein folding, intracellular signalling and enzyme catalysis. 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 [Balla *et al*. 1996, Robeson *et al.* 1980, Rude 1993, Whang *et al.* 1994, Astier *et al.* 1996]. Proteins and lipids damaged by oxygen radicals can no longer normally function. As erythrocytes can not synthesis damaged molecule, repeated damages accelerate their death. It has been shown that erythrocyte from Mg deficient hamster displayed an enhanced susceptibility to oxidative stress [Freedman *et al.* 1992].

There are several interactions between Mg and antioxidant enzymes. Mg is required in processes of transcription and replication of antioxidant enzymes. The increased products of ROS observed during hypomagnesemia may induce structural and functional alteration of enzymes, thus the deficiency of Mg also influence the activity of enzymes [Kuzniar *et al.* 2003]. One mechanism by which Mg deprivation may increase cellular vulnerability to oxidation is by depleting reduced glutathione (GSH) [Freedman *et al.* 1992]. In an oxidative environment there is an increased GSH consumption. Under normal circumstances, the gamma-glutamyl-amino acids generated from GSH are rapidly transported intracellularly and used as substrates for its resynthesis. The two enzymes that catalyze GSH synthesis, glutamate cysteine ligase (formerly called γ-glutamyl-cysteine synthetase) and glutathione synthetase are both Mg-dependent [Minnich *et al.* 1971].

The GSH is significantly depleted in mice kept for more than 3 weeks on Mg-deficient diet with consequent and significant reduction of Mg level in plasma. The pool of GSH was determined to be decreased. The decreased level of GSH may be a consequence of GSH consumption and/or reduced synthesis and transformation to oxidized glutathione (GSSG) and an efflux of GSH from RBCs with damaged membranes [Kuzniar *et al.* 2003].

The ratio of Mg retention to urinary excretion showed a significant inverse correlation with Mg concentration in erythrocytes. Bronchial hyperreactivity had a significant inverse correlation with Mg concentration in erythrocytes [Hashimoto *et al.* 2000].

1.5 *Our aims*

1.5.1 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.

1.5.2. 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.

2. PATIENTS AND METHODS

Subjects and study design

A randomized, double-blind, placebo-controlled 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 (Table 1). 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 (Table 2). The recruited patients for this study exhibited persistent moderate bronchial asthma on the GINA classification.

TABLE 1. Characteristics of patients

	Mg-treated patients	Placebo-treated patients	Total
No. of patients	54	35	89
Boys	40	22	62
Girls	14	13	27
Mild asthmatics	26	16	42
Boys	19	10	29
Girls	7	6	13
Mean age [years]	8.2±2.8	8.7±2.7	
Mean FEV1 [% predicted]	82±1.9	83±1.4	
Daytime symptom score·week^{-1} [0-3]	2.2±0.2	2.3±0.3	
Beta2-agonist use puffs·week^{-1}	2.9±0.7	3.4±0.8	
Moderate asthmatics	28	19	47
Boys	21	12	33
Girls	7	7	14
Mean age [years]	10.8±3.4	8.2±2.9	
Mean FEV1 [% predicted]	69±5.5	72±6.8	
Daytime symptom score·week^{-1} [0-3]	7.8±0.7	7.7±0.6	
Beta2-agonist use puffs/patient ·week^{-1}	30.3±1.6	31.6±2.3	

TABLE 2. Characteristics of patients

	Mg-treated patients (n=24)	Placebo-treated patients (n=16)
Sex (M:F)	17:7	11:5
Age [years]	9.3±0.56	9.1±0.64
FEV1 [% predicted]	75.9±1.8	77.8±1.7
Daytime symptom score·week^{-1} [0-3]	6.3±1.1	4.9±1.3
Beta2-agonist use puffs/patient·week^{-1}	13.6±1.9	17.6±4.1

Parameters are not significantly different between treatment and placebo groups.

The diagnosis of bronchial asthma was based on the clinical, lung function and skin prick tests with 13 common inhalative allergens (Haarlems Allergens Laboratory, The Netherlands) and an elevated serum total IgE level. Scores 0-3 were used by the patients and/or their parents to characterize the severity of their asthma, taking into account daytime symptoms such as coughing, dyspnoea and daytime activity and night-time awakenings because of asthmatic episodes. All the patients increased their forced expiratory volume in one second (FEV1) by at least 15% after 100 μg inhaled Salbutamol. Bronchial hyperreactivity was verified by non-specific bronchial provocation tests (histamine and/or methacholine). Measurement of FEV1 at each visit was chosen to follow up the alterations in airway obstruction [Shingo *et al.* 2001]. The largest FEV1 value from at least three manoeuvres was selected as the best one. Spirometry was performed approximately 10-12 h after the previous dose of either rescue or study medication.

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.

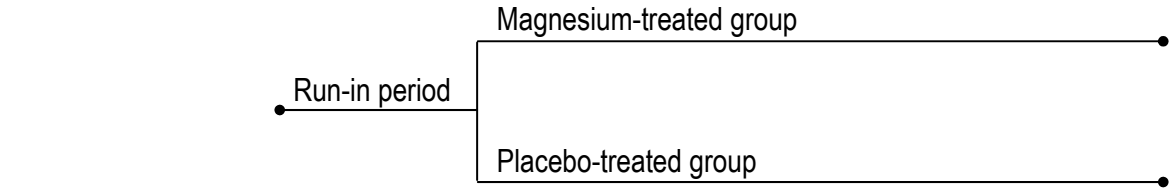
Exclusion criteria included renal failure, malabsorption, hepatic disease, heart failure and arterial hypertension. No child was taking any treatment that affected Mg absorption or excretion, e.g. diuretics, thiazides, digoxin, gentamycin and cyclosporin. All parents and literate patients gave their written consent to participation. This study was approved by the Local Human Investigation Review Board of Albert Szent-Györgyi Medical University.

The investigation ran over three 4-week treatment periods (Figure 2). 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 [54], 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.

At the beginning and end of the 12-week study, venous blood was taken for serum total and free Mg estimation, and blood samples with EDTA and heparin were taken for the investigations listed below. Urine was collected over 24 h for volume and Mg excretion measurements.

FIGURE 2. Schedule of the study



	- 4 weeks	0 week	4 weeks	8 weeks	12 weeks
Written consent	-	+	-	-	-
Inclusion criteria	+	+	-	-	-
Exclusion criteria	+	+	+	+	+
Physical examination	+	+	+	+	+
Lung function (FEV1)	+	+	+	+	+
Blood sample	-	+	-	-	+
24 h urine sample	-	+	-	-	+
Diary card	-	+	+	+	-
Drug dispensation	-	+	+	+	-

Determination of magnesium

Mg forms a coloured complex with xylidyl blue in strongly basic solution, where calcium interference is eliminated by glycol ether diamine-N,N,N'N'-tetraacetic acid. The colour produced, measured biochromatically at 520/800 nm (Hitachi 917

equipment), is proportional to the Mg concentration [Tietz 1990]. 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 µl and measure pH, pCO_2 and pO_2 simultaneously [Thomas 1998].

Determinations of reduced and oxidized glutathione

langnp2057The 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 [Németh *et al.* 1994]. Depending on the concentrations of GSH (µmol/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) [Tietze 1969]. Samples for GSSG were measured after the alkylation of GSH with NEM [Güntherberg *et al.* 1968] and after the separation of GSSG and the NEM-GSH complex by gel filtration with Sephadex G-10 [Akerboom *et a*l. 1981]. The concentrations of GSH and GSSG were expressed with reference to Hb determined by the cyanmethaemoglobin method [Akerboom *et al.* 1981].

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 [Beutler 1957]. The test was applied to measure the capacity of the glutathione redox system to protect Hb after an *in vitro* oxidative stress.

Determinations of oxidized components of haemoglobin from haemolysates

Changes in oxidized components of Hb such as oxyhaemoglobin (oxyHb), methaemoglobin (metHb) and hemichrome from haemolysates were examined before and after *in vitro* incubation with APH by the method of Szebeni 1984. 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 [Winterbourn 1979].

Statistical analysis

The GraphPad Prism 4.00 for Windows software package (GaphPad Software, San Diego California, USA) was used for statistical evaluation. Data are expressed as mean±standard error of the mean (mean±SEM). Parametric data such as total and free Mg levels in the serum and urine, blood Hb, oxyHb, metHb, hemichrome, GSSG, GSH and plasma Hb, oxyHb, metHb, hemichrome and bilirubin, at the beginning and end of the 12-week treatment period, were calculated by using variance analysis and Student's *t*-test.

Non-parametric data such as the numbers of puffs per patient per week of short-term beta$_2$-agonist, alterations in FEV1 and symptom scores during the four periods of 4 weeks, and the ratio GSSG/GSH, and the GSH stability at the beginning and end of the 12-week treatment period were calculated by using the Wilcoxon signed ranks test in the Mg-treated and placebo-treated patients, and the Mann-Whitney test between the Mg-treated and placebo-treated patients. When the variances between the pairs of groups differed significantly from each other ($p<0.05$ in the *F*-test), we used the Welch test (*d*-probe).

Correlations between the plasma Hb and the plasma metHb and hemichrome levels were determined in the Mg-treated and the placebo-treated patients before and after the 12-week treatment period, using linear regression analysis. A p value <0.05 was considered statistically significant.

3. RESULTS

3.1 *Magnesium status*

3.1.1. *Magnesium status in mild asthmatics*

There was a significant increase in serum total Mg at the end of the study in both the Mg and the placebo groups (0.80±0.03 versus 0.89±0.06 mmol/l, $p<0.01$ and 0.75±0.04 versus 0.82±0.07 mmol/l, $p=0.01$, respectively). The mean serum total Mg was significantly higher at the end in the Mg group than in the placebo group (0.89±0.06 versus 0.82±0.07 mmol/l, $p<0.05$).

There was a statistically significant decrease in serum free Mg at the end of the Mg treatment period (0.56±0.03 versus 0.53±0.02 mmol/l, $p<0.01$) without clinical consequences. There was no change in serum free Mg at the end of the placebo treatment period (0.51±0.03 versus 0.52±0.04 mmol/l, *p*=NS).

The 24-hr Mg excretion was not significantly increased at the end of either the Mg or the placebo treatment period (3.54±1.50 versus 4.54±1.90 mmol/day, *p*=NS and 4.04±1.16 versus 5.13±2.79 mmol/day, *p*=NS, respectively).

3.1.2. *Magnesium status in moderate asthmatics*

The initial mean±SD serum total Mg levels in the Mg and placebo groups were 0.80±0.03 and 0.79±0.04 mmol/l, respectively. At the end of the study, there were significant increases in serum total Mg in both the Mg and the placebo groups (0.88±0.05 mmol/l, $p<0.01$ and 0.85±0.05 mmol/l, $p<0.05$, respectively).

There was no significant change in serum free Mg at the end of the Mg treatment (0.54±0.02 versus 0.54±0.03 mmol/l, *p*=NS), similarly as for the placebo-treated patients (0.53±0.03 versus 0.51±0.03 mmol/l, *p*=NS).

There was a significant increase in the mean 24-hr urine Mg excretion at the end of the Mg treatment (4.57±1.90 versus 6.81±3.90 mmol/day, $p<0.05$), and a significant decrease at the end of the placebo treatment (4.13±3.53 versus 2.79±1.39 mmol/day, $p=0.01$). The mean 24-hr urine Mg excretion was significantly higher after the Mg

treatment than after the placebo treatment (6.81±3.90 versus 2.79±1.39 mmol/day, $p<0.05$).

3.2. *Lung function*

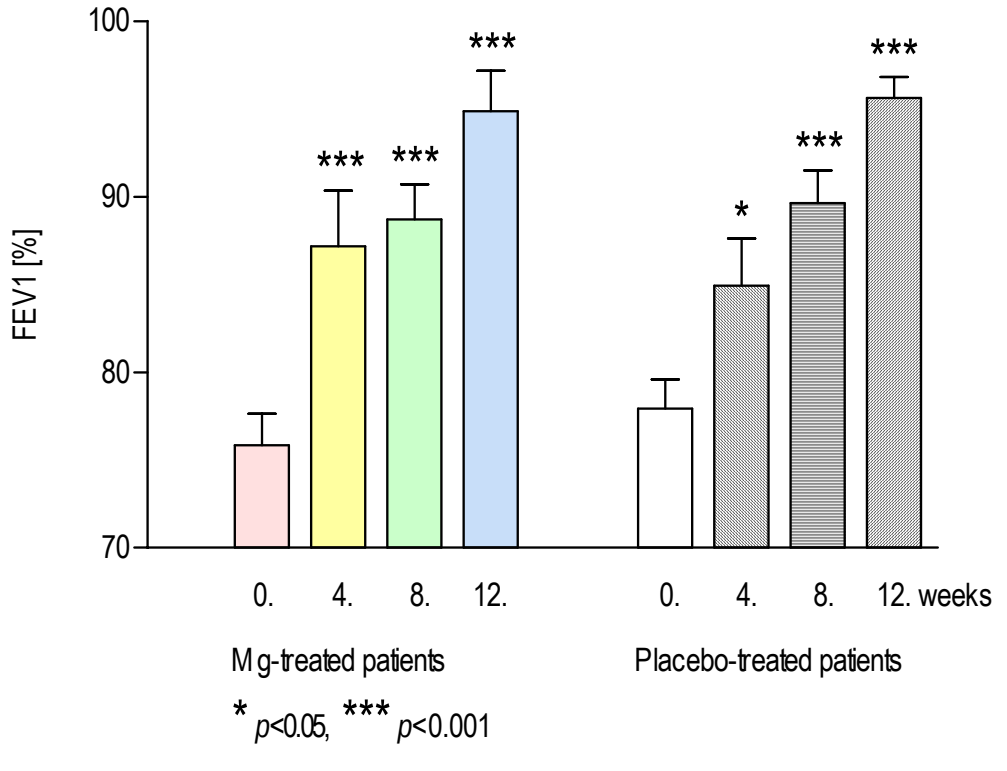
3.2.1 *Changes in FEV1 in mild asthmatics*

There was a significantly increase FEV1 at the end of the study in both the Mg-treated and the placebo-treated patients (82±1.9 versus 89±9.3%, $p<0.05$ and 83±1.4 versus 86±3.4%, $p<0.05$, respectively).

3.2.2. *Changes in FEV1 in moderate asthmatics*

Almost in parallel a gradual significant increase in FEV1 was observed in both the Mg-treated and the placebo-treated groups (Figure 3).

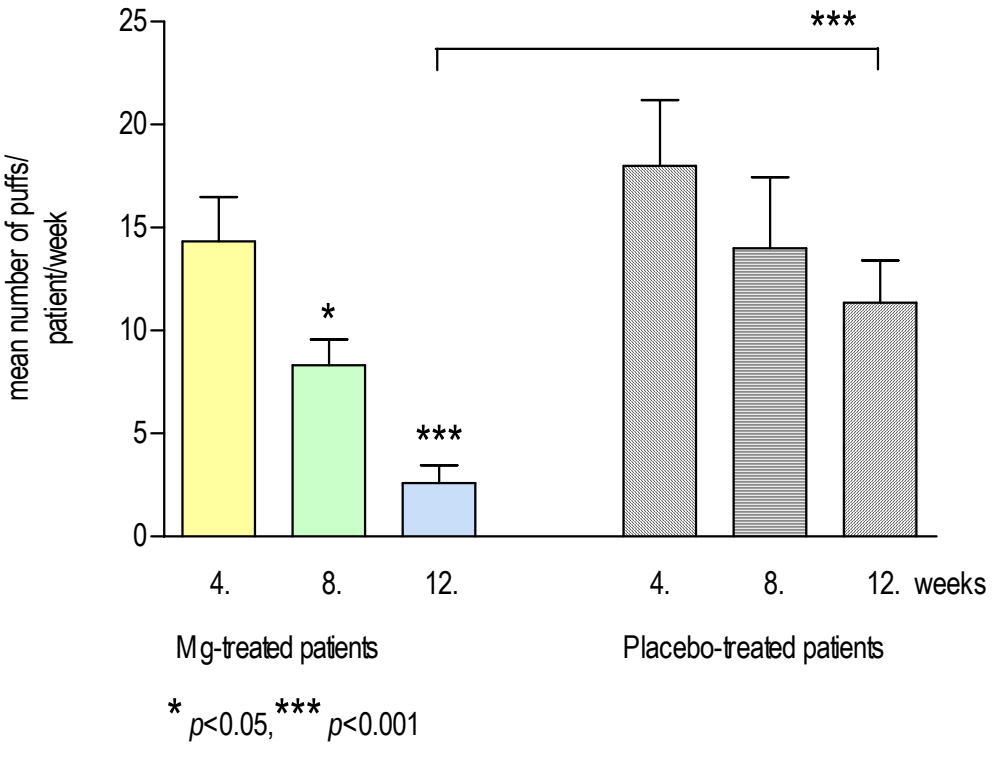
FIGURE 3. Changes in FEV1 during a 12-week treatment period in patients with moderate asthma (mean±SEM)



3.3. *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 (Figure 4).

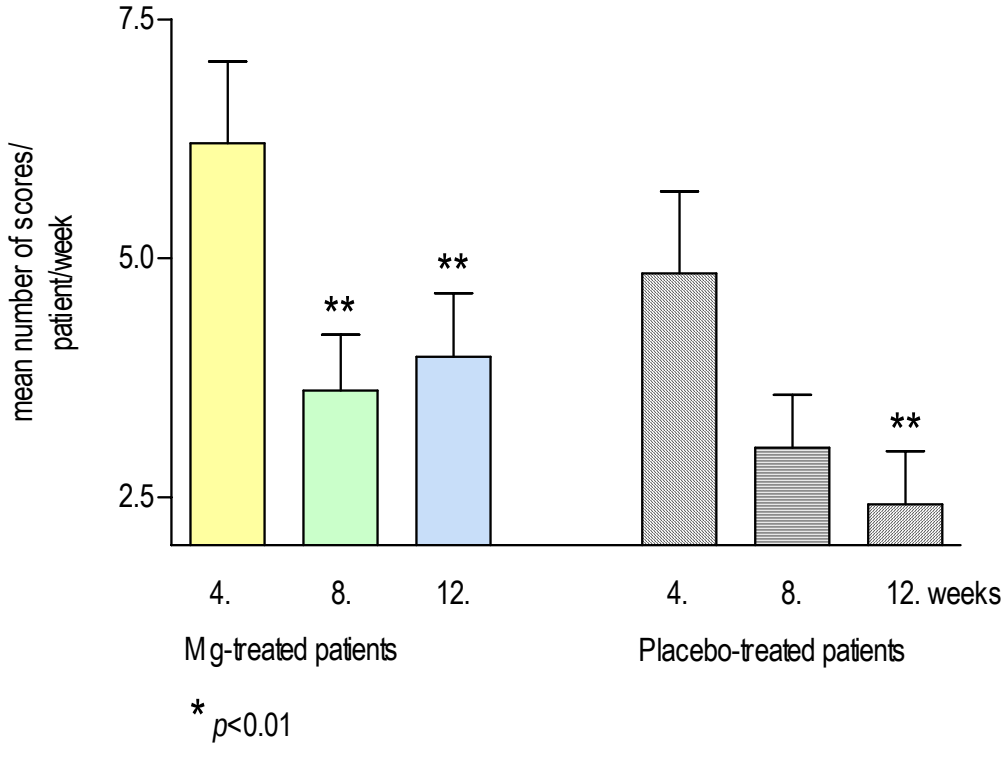
FIGURE 4. Changes in number of puffs / patient /week of beta2-agonist used during a 12-week treatment period in patients with moderate asthma (mean±SEM)



3.4. *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 (Figure 5).

FIGURE 5. Changes in total daytime score during a 4-week treatment period in the patients with moderate asthma (mean±SEM)

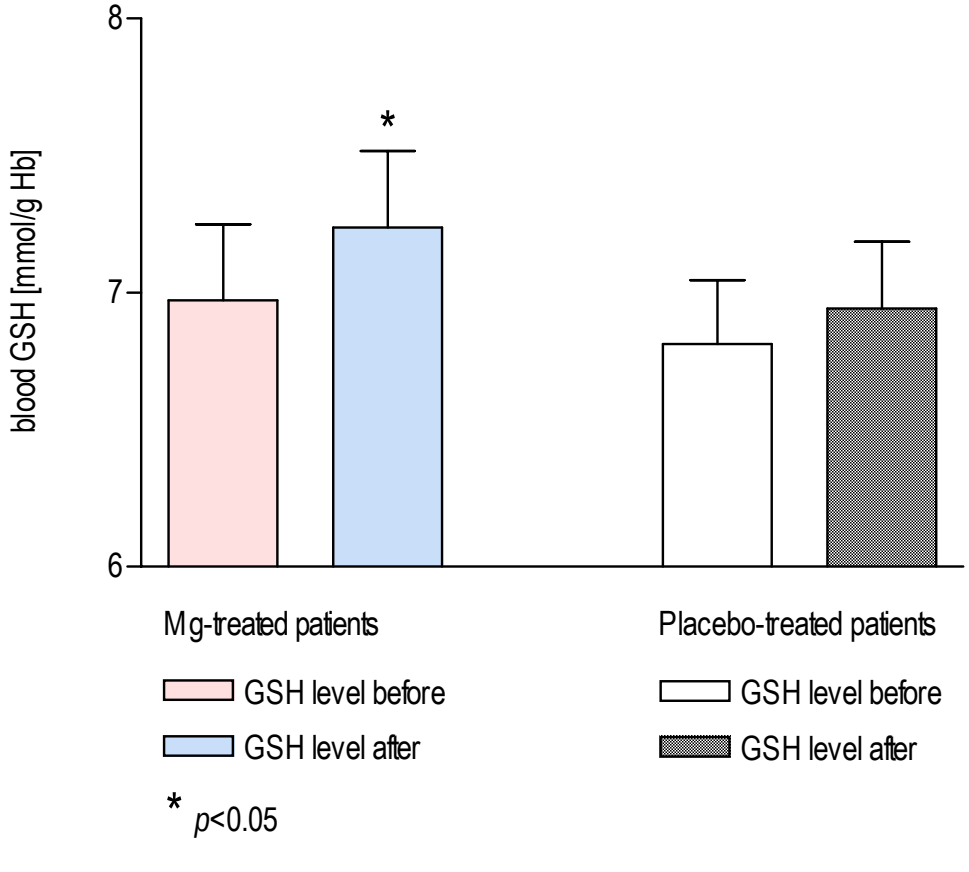


3.5. *Changes in redox system*

3.5.1. *Reduced and oxidized glutathione in the blood*

There was a significant increase in GSH concentration ((6.97±0.28 μmol/g Hb (95% CI: 6.40-7.55) versus 7.24±0.28 μmol/g Hb (95% CI: 6.66-7.82), $p<0.05$)) in the Mg-treated patients after the 12-week treatment period, but not in the placebo-treated group ((6.81±0.23 μmol/g Hb (95% CI: 6.32-7.31) versus 6.94±0.24 μmol/g Hb (95% CI: 6.42-7.46), *p*=NS), Figure 6). The GSSG concentration did not show any significant alterations during the 12-week treatment period in either the Mg-treated ((0.007±0.001 nmol/g Hb (95% CI: 0.005-0.009) versus 0.007±0.001 nmol/g Hb (95% CI: 0.006-0.009), *p*=NS)) or the placebo-treated patients ((0.01±0.001 nmol/g Hb (95% CI: 0.007-0.012) versus 0.007±0.001 nmol/g Hb (95% CI: 0.005-0.009), *p*=NS)).

FIGURE 6. Changes in GSH levels in the Mg and the placebo-treated patients before and after a 12-week treatment period (mean±SEM)



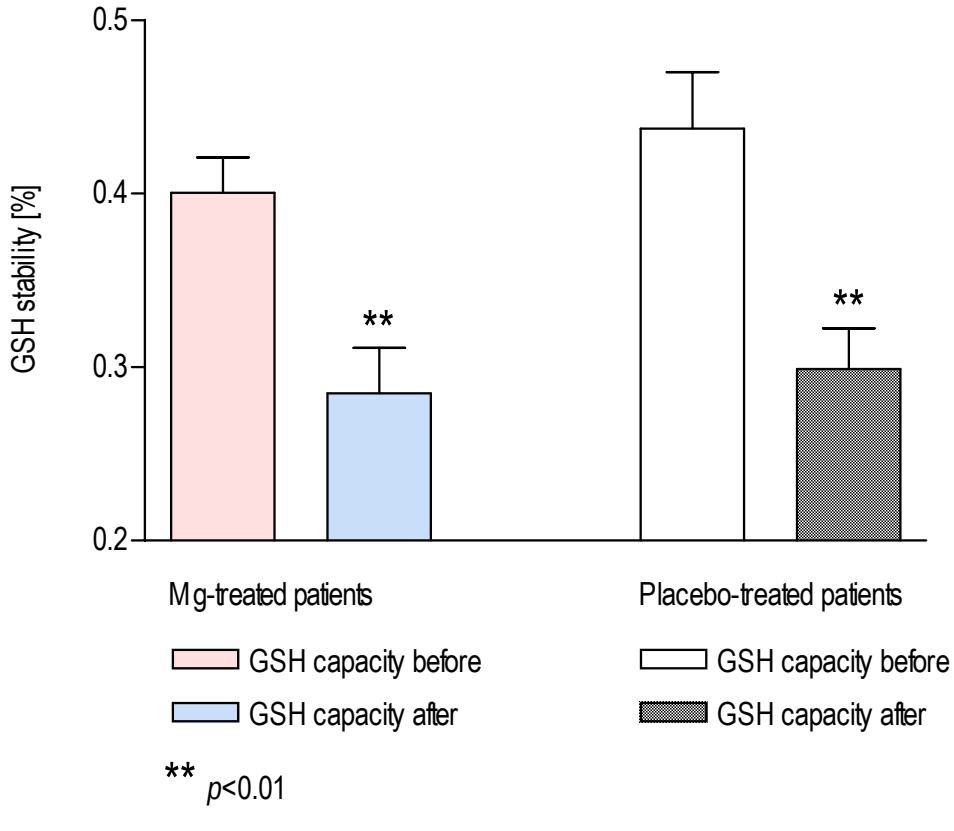
3.5.2. *Glutathione redox ratio*

A significant decrease in the molar ratio GSSG/GSH ((13.73±1.52% (95% CI: 10.50-16.96) versus 9.64±1.13% (95% CI: 7.23-12.05), $p<0.05$)) was observed in the placebo-treated patients after 12 weeks, but not in the Mg-treated patients ((13.40±2.17% (95% CI: 8.78-18.02) versus 9.50±1.39% (95% CI: 6.57-12.43), p=NS)).

3.5.3. *Reduced glutathione stability test*

The GSH stability test (Figure 7) revealed that the residual capacity of the RBCs after an *in vitro* challenge with APH was significantly lower in both the Mg-treated and the placebo-treated groups ((40.04±2.03% (95% CI: 35.84-44.25) versus 28.50±2.03% (95% CI: 23.10-33.90), $p<0.01$ and 43.75±3.26% (95% CI: 36.80-50.70) versus 29.88±2.37% (95% CI: 24.83-34.92), $p<0.01$, respectively)).

FIGURE 7. Residual GSH capacity [%] after an APH challenge in the Mg and the placebo-treated patients before and after a 12-week treatment period (mean±SEM)



3.5.4. *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 values measured at 630 nm, the hemichrome values measured at 700 nm and the bilirubin values measured at 460 nm were significantly decreased to similar extents in both groups, as shown in Table 3.

TABLE 3. Changes in oxyhaemoglobin, methaemoglobin, hemichrome and bilirubin concentrations in the plasma before and after the 12-week treatment period (mean±SEM)

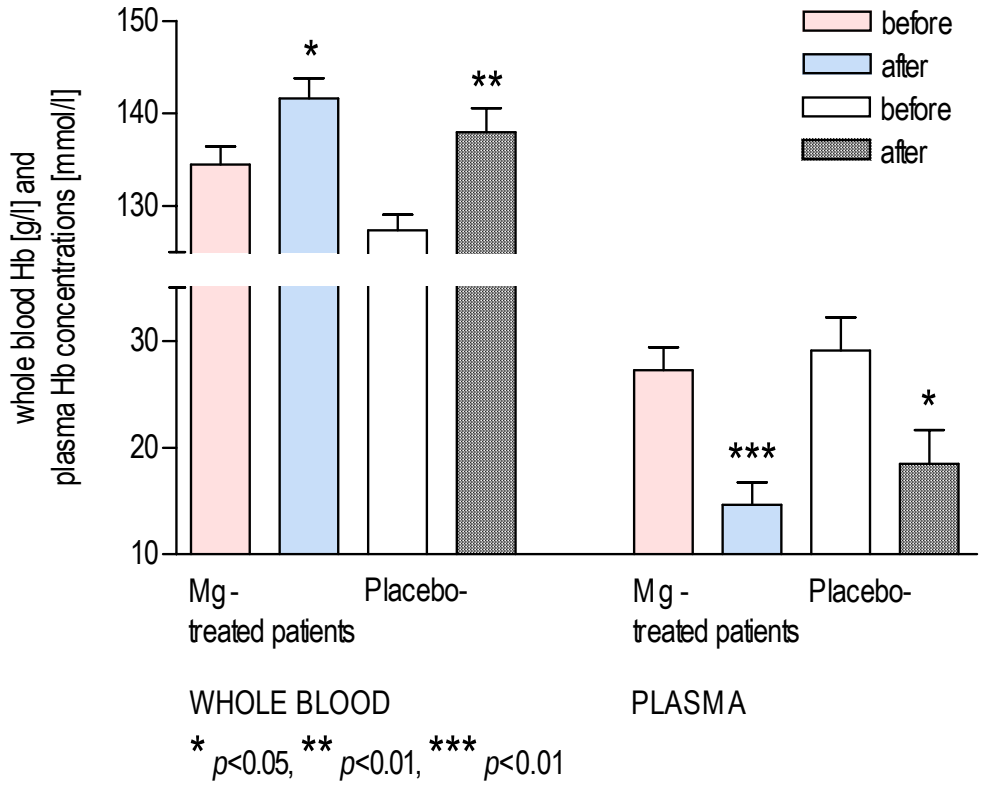
	Mg-treated patients (95% CI)	Placebo-treated patients (95% CI)
Oxyhaemoglobin [μmol/l]		
Before	10.68±2.23 (6.07-15.29)	11.82±2.61 (6.26-17.38)
After	6.79±1.81 (3.05-10.54)	10.40±2.96 (4.10-16.70)
Methaemoglobin [μmol/l]		
Before	43.46±4.37 (34.41-52.50)	46.36±4.28 (37.23-55.49)
After	12.97±3.53 (5.66-20.28) ***	12.99±5.94 (0.33-25.65) ***
Hemichrome [μmol/l]		
Before	33.50±3.64 (25.97-41.04)	35.40±4.03 (26.82-43.98)
After	13.04±1.81 (9.30-16.79) ***	12.28±3.46 (4.90-19.65) ***
Bilirubin [mmol/l]		
Before	29.35±1.72 (25.80-32.90)	29.89±1.74 (26.19-33.58)
After	16.39±1.13 (14.06-18.71) ***	17.49±1.52 (14.24-20.74) ***

Asterisks denote significant differences between the data for the Mg-treated and the placebo-treated patients before and after treatment. *** $p<0.001$

3.5.5. *Whole blood and plasma haemoglobin concentrations*

The alterations in the whole blood and plasma Hb concentrations before and after the 12-week treatment period are to be seen in Figure 8. The decrease in the plasma Hb levels was significant 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.

FIGURE 8. Changes in haemoglobin concentration in whole blood and plasma before and after a 12-week treatment period in the Mg-treated and placebo-treated patients (mean±SEM).



3.5.6. *Whole blood oxyhaemoglobin, methaemoglobin and hemichrome concentrations*

Measurements of the whole blood oxyHb, metHb and hemichrome concentrations in the haemolysates 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 ((Mg group: 12.36±0.68% (95% CI: 10.96-13.76) versus 9.74±0.88% (95% CI: 7.92-11.56), $p<0.05$ and placebo group: 13.91±1.56% (95% CI: 10.58-17.23) versus 10.30±0.72% (95% CI: 8.77-11.83), $p<0.05$, respectively)).

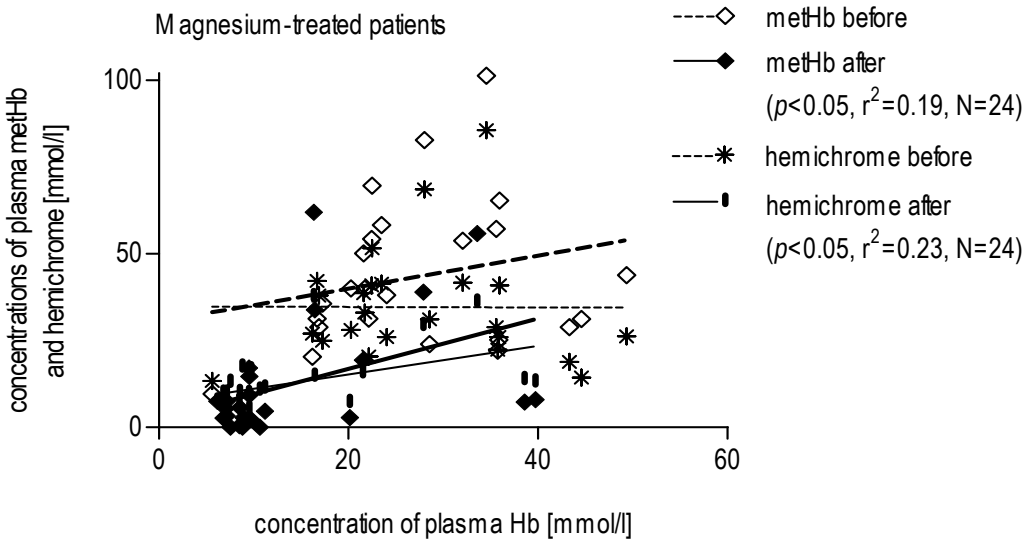
The metHb concentration was considerably higher in the Mg-treated patients after acute oxidative stress than in the placebo-treated patients ((59.81±0.42% (95% CI: 58.95-60.67) versus 61.08±0.84% (95% CI: 59.35-62.81), $p<0.05$ and 58.14±0.84% (95% CI: 56.35-59.93) versus 59.46±0.31% (95% CI: 58.80-60.12), p=NS, respectively)).

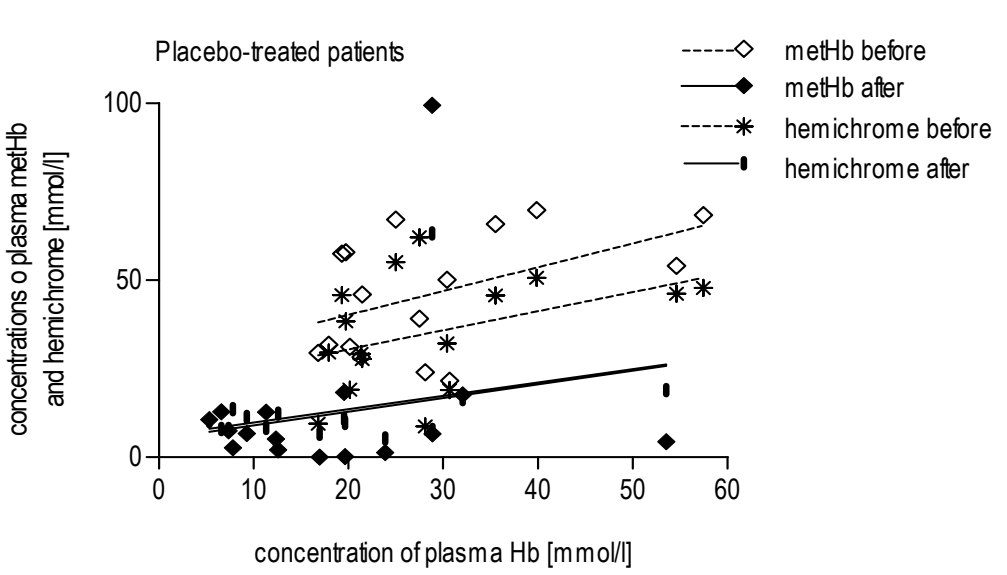
There was no significant alteration in the hemichrome concentrations before and after the treatment period in either group ((Mg group: 27.83±0.69% (95% CI: 26.41-29.26) versus 29.17±0.42% (95% CI: 28.30-30.04), p=NS and placebo group: 27.95±0.95% (95% CI: 25.93-29.97) versus 30.24±0.69% (95% CI: 28.77-31.72), p=NS)).

3.5.7. *Linear regression between plasma haemoglobin concentration and plasma methaemoglobin and hemichrome 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 (Figure 9).

FIGURE 9. Analysis of linear regression between the plasma Hb and metHb and hemichrome concentrations before and after a 12-week treatment period.





4. DISCUSSION

4.1. *Magnesium deficiency*

Magnesium deficiency is now considered to contribute to many diseases. Specific clinical condition in which Mg deficiency has been implicated to play a pathophysiological role is among others bronchial asthma. The average Westernized diet is sufficient to prevent Mg deficiency but does not appear to provide enough Mg to maintain high normal serum Mg levels that may be protect against diseases [Vormann 2002].

Magnesium has beneficial effects on the airways. Individuals with low Mg intake may be at higher risk of developing asthma. A large epidemiological study showed that a lower dietary Mg intake in general population is associated with impaired lung function, bronchial hyperreactivity and increased risk of wheezing [Britton *et al.* 1994]. Poor Mg intake is associated with impairment of pulmonary function, determined by a decreased in forced expiratory volume in one second (FEV1) and a higher risk of both wheezing and airway hyperreactivity, especially in childhood [Hijazi *et al.* 2000, Baker *et al.* 2000].

It seems that the two types of Mg deficit may coexist in asthmatic children. Mg deficiency caused by an inappropriate Mg intake can be treated by oral physiological Mg supplementation, but Mg depletion resulting from disturbances of the control mechanisms of the Mg metabolism can not be solved by simple oral supplementation [Durlach *et al.* 1995, 2005]. The organism has the task of maintenance of the homeostasis of the normal Mg status in both extracellular and intracellular compartments. It may explain that no alteration in the serum Mg level was observed during asthmatic attacks or histamine and methacholine challenge [Kakish 2001, Zervas *et al.* 2000]. A reduced erythrocyte levels were observed during asthmatic attack or histamine and methacholine challenge [Zervas *et al.* 2000, Emelyanov *et al.* 1999], whereas they normalized in symptom-free period [Fantidis *et al.* 1995].

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 at the beginning of the study. The mean serum total Mg was significantly increased in both the treated and the placebo groups at the end of the study.

4.2. *Magnesium depletion*

The Mg deficit can result from both an insufficient Mg intake and also from alterations in Mg retention mechanisms. When different stresses transform the Mg deficiency into Mg depletion related to a dysregulation of the control of Mg status, nutritional physiological Mg supplementation alone is ineffective. In addition, beta2-agonists which are the first line of asthma therapy can stimulate Mg efflux in peripheral tissues, leading to an aggravated Mg deficit of the cells [Khilnani *et al.* rsid14490531 1992, Durlach *et al.* 2003]. A low skeletal Mg content was also reported in stable asthmatics treated with oral beta2-agonists [Gustafson *et al.* 1996].

The Mg deficit in the body stores was revealed by a reduced erythrocyte Mg concentration in some stable asthmatic adults, with a significant inverse correlation between the urinary Mg retention and the erythrocyte Mg concentration. The ratio of Mg retention to urinary excretion and bronchial reactivity to inhaled methacholine was significantly inversely correlated with the erythrocyte Mg level [Hashimoto *et al.* 2000]. In consequence, the greater the urinary Mg retention, the greater the intracellular Mg deficit may be as proved by measurement of the urinary Mg concentration. The daily urine Mg excretion in a severe Mg deficiency may be less than 0.5 mmol whereas in a mild Mg deficiency is less than 2.7 mmol. Hypomagnesemia (serum total Mg level<0.74 mmol/l) is almost always associated with more severe asthma, while patients with mild or moderate asthma have normal Mg levels [Alamoudi 2000].

A significant decrease in the 24-h urine Mg excretion, associated with normal total and free Mg levels in the serum, was seen at the end of the 12-week period in the placebo-treated moderately asthmatic children but not in the mild asthmatics. It seemed that the normal diet provided an appropriate Mg intake in the mildly, but not the moderately asthmatic children. This was confirmed by the non-significant increase in urinary Mg excretion in the mild asthmatics.

Thus, in asthmatics the Mg deficit can be characteristic of alterations in Mg retention mechanisms and of an appropriate Mg intake as well. Accordingly, a mild Mg deficiency resulting from an insufficient Mg intake develops in placebo-treated children with moderate asthma. In addition, beta2-mimetic use proved significantly more frequent in placebo-treated than in Mg-treated moderately asthmatic children. Beta-adrenergic agonists can stimulate Mg efflux in peripheral tissues, which can aggravate the Mg deficit of the cells indicating a certain degree of Mg depletion.

4.3. *Magnesium supplementation, lung function and clinics*

A 200-600 mg/day Mg intake was earlier found to be associated with a higher FEV1 and a reduction in bronchial hyperreactivity [Britton *et al.* 1994, Gontijo-Amaral *et al.* 2007]. A short-term Mg intake of 400 mg/day can improve clinical symptoms in adults [Hill *et al.* 1997]. The recommended dietary Mg intake is 350 mg/day for a male adult, 280 mg/day for a female and 10-13 mg/kg/day for growing children [54]. During the past three decades, it was emphasized that the Mg requirement of almost all healthy adults is 6 mg/kg/day [Durlach 2001]. We administered 200 mg/day Mg for ≤7-year-old children and 290 mg/day to older children, which was considered sufficient for children on a normal diet.

Mg supplementation associated with a lower requirement of short-term inhaled beta2-mimetics was followed by a higher FEV1 from the early phase of the study in the Mg-treated patients with moderate asthma. 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 the placebo-treated patients with moderate asthma. The result of frequently used beta2-agonists was the improvement of lung function in the placebo-treated patients.

Nutritional Mg supplementation brings on specific reversibility of the symptoms of asthma causing an improvement of asthma symptom scores [Hill *et al.* 1997, Britton *et al.* 1994]. The daytime symptoms were already significantly reduced at 8 weeks in the Mg-treated patients with moderate asthma. Improvement of the clinical symptoms can be considered more moderate.

There were no side-effects or adverse events during the 12-week period. Our study demonstrated that a large oral dose of Mg (with an atoxic nature) did not cause any

disturbances. The Mg requirements may be greater in growing children, especially those with asthma.

4.4 *Glutathione redox system*

Biological systems are continuously exposed to oxidants, either generated endogenously by metabolic reactions or exogenously, such as air pollutions, infections or allergens. The oxidant burden in the lungs is enhanced by the release of ROS from macrophages, neutrophils and other cells [Rahman *et al.* 1996, 2005]. Reactive oxygen species, such as the superoxide and the hydroxyl radical, are highly unstable species, capable of initiating oxidation. ROS causes oxidation of proteins, DNA and lipids which may cause direct lung injury or induce a variety of cellular responses or may alter remodelling of extracellular matrix, cause apoptosis and regulate cell proliferation [Rahman 2005]. Since the 1990s ROS has been implicated in initiating inflammatory responses in the lungs trough the activation of transcription factors and other signal transduction pathways leading to enhanced gene expression of proinflammatory mediators [Kamata *et al.* 2005]. Recently, it has been shown that oxidative stress and the redox status of the cells can also regulate nuclear histone modifications, such as acetylation, methylation and phosphorylation leading to the induction of proinflammatory mediators [Barnes *et al.* 2005].

The environment and inflammatory cells are a major source of ROS. GSH is the most important nonprotein sulfhydryl in the cells and play a key role in the maintenance of the cellular redox status. The redox potential is defined as the ratio of the concentration of oxidizing equivalents to that of reducing equivalents [Forman *et al.* 2003]. Two major redox forms of glutathione have been identified in the cells, i.e. reduced glutathione and glutathione disulphide. GSSG represents a negligible fraction (1/100th) of the total GSH pool. *De novo* synthesis of GSH its amino acid constituents are essential for elevation of GSH that occurs as an adaptive response to oxidative stress. GSH synthesis involves two enzymatic steps catalysed by glutamate cysteine ligase (formerly called γ-glutamyl cysteine synthetase) and glutathione synthetase, which are both Mg-dependent [Huang *et al.* 1993, Minnich *et al*. 1971].
Mg deficiency could promote the action of calcium. It has been demonstrated that the action of prostanoids, which amplified the airway smooth muscle contractions [Hardy 1984], is Mg-dependent and that tissue levels of prostanoids are higher in Mg-deficient rats [Altura *et al.* 1976].

The antioxidant properties of Mg have been extensively characterized. Rodents fed a low Mg diet had evidence of lipid peroxidation in a variety of organs, including heart, liver, skeletal muscle and testis [Rayssiguier *et al.* 1993]. Consistent with these observations, tissues derived from Mg-deficient animals were highly vulnerable to peroxidation in vitro. One mechanism by which Mg deprivation may increase cellular vulnerability to oxidation is by depleting reduced glutathione [Freedman *et al.* 1992, Mills *et al.* 1986, Wiles *et al.* 1996].

The RBCs can be regarded as circulatory antioxidant carriers which reflect exposure to ROS [Kappus *et al.* 1981, Malmgren *et al.* 1986]. Since RBCs are available easier than the lung tissue and play the most essential role in the antioxidant defence mechanisms, they have been used in the evaluation of the degree of oxidative stress [Németh *et al.* 1994]. Two metabolic pathways can occur inside the RBCs: the anaerobic Embden-Meyerhof pathway, which utilizes about 90% of the glucose to generate high-energy phosphates, and the oxidative hexose monophosphate pathway (HMP), which utilizes a small amount of glucose to defend against oxidative effects on Hb and cellular proteins. HMP shunt is associated with the generation of NADPH can catalyse the formation of GSH in response to an acute oxidant challenge, causing a rapid increase in GSSG concentration, as previously proven in certain diseases [Paglia 1995]. Chronic oxidative stress was characterized by a low GSSG concentration, a reduced ratio GSSG/GSH and a lower residual GSH capacity in asthmatic children at the beginning of the study as compared with the corresponding normal data reported in the literature.

The GSH concentrations were significantly increased in the Mg-treated patients after the treatment period, but not in the placebo-treated patients, demonstrating either the presence of chronic oxidative stress or the fact that other compensatory mechanisms serving the defence against oxidative agents are activated to maintain the normal GSH level. The most sensitive indicator of acute oxidative stress is an increase in the molar ratio GSSG/GSH, which was not observed in the Mg-treated patients, indicating that neither acute nor chronic oxidant events were able to worsen the patients' conditions. A significant decrease in GSSG/GSH, indicative of the occurrence of chronic oxidative stress associated with an insignificant decrease in GSSG concentration and a poorly increased GSH concentration, was found in the placebo-treated asthmatics. The ability to maintain the normal GSH concentration

was not observed in the placebo-treated asthmatics. Significantly decreased residual GSH concentrations and an impaired GSH recycling capacity due to the permanent oxidative stress may be the cause.

The residual GSH ratio determined in the GSH stability test was approximately 42% before the treatment period. After the induction of *in vitro* oxidative stress with APH, the residual GSH concentration exhibited a significant decrease at the end of the study in both groups, as a sign of the increased sensitivity and the decreased antioxidant capacity of patients to resist an oxidative challenge. Mg supplementation in the given doses did not improve the residual GSH capacity in asthmatics.

4.5. *Oxidative haemolysis*

Because of the lower GSH capacity, acute oxidative stress induced with APH after the 12-week Mg supplementation revealed the enhanced oxidation of Hb, resulting in a significant decrease in oxyHb, a significant increase in metHb and an unchanged hemichrome concentration in the whole blood in the Mg-treated patients, but not in the placebo-treated patients (except for the metHb level which was unchanged and the hemichrome level which displayed an increasing tendency). This is probably due to the later phase of persistent oxidative stress producing more hemichrome, with membrane-damaging properties. Mg seems to exert a functional protective capacity on generating hemichrome.

An insufficient Mg supply can cause a defective glucose metabolism in the RBCs (the activity of hexokinase catalysing the formation of glucose-6-phosphate is a Mg-dependent process), leading to reduced concentrations of ribulose-5-phosphate and NADPH in the hexose monophosphate shunt. In the absence of NADPH, the reduction of GSSG is damaged causing a decreased production of GSH as was observed in the placebo-treated asthmatics. One possible explanation for these observations may be that GSH was consumed in an oxidative intracellular environment, converted to GSSG and released from the cell. The consumption of GSH is not predominant because the concentration of GSSG was decreased but not significantly in the placebo-treated patients. Under normal conditions, *de novo* GSH synthesis may compensate for this loss, resulting in an increased turnover. In Mg deprivation this synthesis is decreased due to the inactivation of the two enzymes that catalyze GSH synthesis [Minnich *et al.* 1971]. Moderation of the oxidative

stress, restoration of the saturation of the Mg stores in consequence of a reduced requirement for Mg and other compensatory mechanisms can afford an adequate explanation for the increased Mg excretion at the end of the 12-week treatment period in the Mg-treated patients.

The differentially reduced plasma Hb concentrations in both groups may indicate the presence of chronic oxidative stress and the defective recycling of GSH in the placebo-treated patients and the intact recycling of GSH in the Mg-treated group. 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. Oxidative stress causing haemolysis may generate more and more metHb and hemichrome, depending on the Hb concentration in the RBCs. Mg supplementation in the given doses effectively influenced this process in the Mg-treated patients, but not in the placebo-treated patients.

Mg plays a crucial role in numerous biological processes as a cofactor in hundreds of enzymatic reactions in the body [Romani *et al.* 1992], e.g. in oxidative glycolysis. In the absence of Mg, the activity of the antioxidant system may be damaged. Additionally, the excessive use of beta2-agonists may induce a decrease of magnesemia causing depletion of the activation of the GSH/GSSG redox system. Beta2-agonists are able to mobilize Mg from stores to maintain the normal Mg homeostasis [Durlach *et al*. 2003, Khilnani *et al.* 1992]. The bronchial asthma in the placebo-treated children was characterized by significantly more exacerbations requiring the administration of beta2-agonists significantly more often as needed medication.

There were no side-effects or adverse events during the 12-week period. Our study demonstrated that a large oral dose of Mg (with an atoxic nature) did not cause any disturbances, improved the magnesium status and showed an antioxidant activity. The Mg requirements may be greater in growing children, especially those with asthma.

5. 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 simply 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 metHb 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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SUPPLEMENTS