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Original Paper

Protective Role of Magnesium against Oxidative Stress on $SO_4^=$ Uptake through Band 3 Protein in Human Erythrocytes

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Key Words
Magnesium • Band 3 protein • $SO_4^=$ uptake • Hydrogen peroxide • N-ethylmaleimide

Abstract
Background/Aims: Magnesium, whose supplementation provides beneficial effects against oxidative stress-related conditions, has been here used to possibly protect Band 3 protein anion exchange capability and underlying signaling in an *in vitro* model of oxidative stress. ***Methods:*** Whole blood samples pre-exposed to 10 mM $MgCl_2$, were treated for 30 min with H_2O_2 (300 μM, 600 μM and 1 mM) chosen as oxidant molecule. In a separate protocol, NEM (0.5,1 and 2 mM), a phosphatase inhibitor and thiol-alkilant agent, has been also applied. The rate constant for $SO_4^=$ uptake, accounting for Band 3 protein anion exchange capability, has been measured by a turbidimetric method, while intracellular reduced glutathione (GSH) levels and membrane –SH groups mostly belonging to Band 3 protein were spectrophotometrically quantified after reaction with DTNB (5,5'-dithiobis-(2-nitrobenzoic acid). Expression levels of Band 3 protein, phosporylated Tyrosine (P-Tyr) and tyrosine kinase (Syk) involved in signaling have been also measured. ***Results:*** Our results show that Mg^{2+} prevented the reduction in the rate constant for $SO_4^=$ uptake on H_2O_2-treated erythrocytes, not involving GSH levels and membrane -SH groups, unlike NEM, remaining both P-Tyr and Syk expression levels high. ***Conclusion:*** Hence, i) the measurement of the rate constant for $SO_4^=$ uptake is a useful tool to evaluate Mg^{2+} protective effect; ii) the use of two different oxidant molecules shed light on Mg^{2+} effect which seems not to modulate phosphorylative pathways but would putatively stabilize membrane organization; iii) the use of Mg^{2+} in food supplementation can be reasonably supported to protect erythrocytes homeostasis in case of oxidative stress-related diseases.

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Introduction

Magnesium, the second most abundant intracellular ion after K^+ with concentrations ranging between 10 and 30 mM, modulates cell volume regulation, enzymes activity and erythrocytes membrane physical properties [1-3]. Benefits of Mg^{2+} supplementation have been shown in preeclampsia, arrhythmia, severe asthma, migraine and in case of improved glucose and insulin metabolism, alleviated dysmenorrhea and leg cramps in women [1, 4]. Moreover, a beneficial effect of Mg^{2+} against the risk of fetal hypoxia has been also reported [5]. Though these authors [5], along with Teti et al. and De Luca et al. [6, 7], demonstrated a relationship between Mg^{2+} and ion transport, with specific regard to Band 3 protein in human erythrocytes, the protective role of this metal against oxidative events remains still underexplored+[1, 8-10]. Band 3 protein is the most abundant integral membrane protein of human erythrocytes and, as involved in gas exchange, ion balance, membrane deformability, is essential to erythrocytes homeostasis [8, 9,11, 12]. It is considered as the fastest chloride transporter [13] and, being sulphate ($SO_4^=$) more slowly exchanged through this transporter, the rate constant for $SO_4^=$ uptake effectively accounts for Band 3 protein efficiency [13]. Erythrocytes membrane is often exposed to oxidative conditions, resulting in oxidation of membrane lipids and proteins [14]. Reactive oxygen species (ROS), impacting on erythrocytes membrane, may derive from denatured hemoglobin species in thalassemia and sickle cell anemia [15], G6PD deficiency [16], inflammation or drugs and foods [8]. *In vitro* models of oxidative stress have already demonstrated that Band 3 protein efficiency is compromised by oxidants, such as N-ethylmaleimide (NEM), a thiol-alkilant and phosphatase inhibitor [6, 17, 18]. In particular, as reported by Teti et al. [6], NEM induces a decrease in anion exchange capability through Band 3 protein putatively *via* –SH groups oxidation, phosphorylation of Tyrosine residues of Band 3 protein [17-20] and activation of K-Cl cotransport (KCC), with consequent cell shrinkage and Band 3 protein function impairment. In addition, methemoglobin production and spectrin-hemoglobin (Hb) complexes, affecting anion exchange capability through Band 3 protein, have been also proven [17, 19]. Based on this mechanism of action, NEM has been here considered to prove the antioxidant effect of Mg^{2+}[1, 8-10]. In addition, to compare the effect of two different oxidant molecules and possibly verify Mg^{2+} mechanism of action, an *in vitro* H_2O_2-induced oxidative stress model has also been used [20]. Oxidative damage due to both H_2O_2 and NEM, along with the possible protective effect of Mg^{2+}, have been assessed by determining membrane –SH groups, intracellular levels of reduced glutathione (GSH), $SO_4^=$ uptake velocity through Band 3 protein and Band 3 protein expression levels. Furthermore, phosphorylative transduction pathways, i.e. expression levels of phosphorylated Tyrosine (P-Tyr) and Syk kinase underlying Band 3 protein function and critically involved in Band 3 protein response to oxidative stress [21-23], have been evaluated. Our hypothesis is that the reduced efficiency of $SO_4^=$ uptake through Band 3 protein, due to oxidative stress [17, 20], can be prevented or attenuated by 10 mM Mg^{2+} pre-incubation. Mg^{2+} concentration, though higher than that one used by Teti et al. [6], falls into the range of concentrations used as supplementation in clinical practice [5], as reviewed by Nattagh Eshtivani et al. [24]. The present study may provide novel elements to support the role of Mg^{2+} as an antioxidant food supplement, namely in case of oxidative stress-related diseases which, as already described, may affect Band 3 protein efficiency [25-28].

Materials and Methods

Solutions and chemicals

All chemicals were purchased from Sigma (Milan, Italy). H_2O_2 dilutions were freshly obtained with distilled water from 30% w/w stock solution . $MgCl_2$ stock solution (1 M) was prepared in distilled water. DIDS (4, 4'-diisothiocyanato-stilbene-2, 2'-disulfonate) stock solution (10 mM) was prepared in DMSO. . NEM (N-ethylmaleimide) stock solution (1 mM) was prepared in ethanol.

Erythrocytes preparation

Human blood was obtained from healthy volunteers upon informed consent and according to Ethics Committee guidelines. Blood was collected in heparinized tubes and divided into two aliquots addressed to experimental protocols described below, with or without 10 mM Mg^{2+} pre-exposure. Erythrocytes not exposed to Mg^{2+} are referred to as untreated erythrocytes. Whole blood samples (1 ml), with or without Mg^{2+}, were incubated for one hour at 37 °C with gentle shaking and then addressed to GSH assay (see below).

With regard the other tests, whole blood, pre-incubated or not with 10 mM Mg^{2+}, was washed with an isotonic solution and centrifuged (1200 g, 5 min) to remove plasma and buffy coat. After this operation, repeated thrice, erythrocytes were suspended to either 3 % or 10 % concentration for $SO_4^=$ uptake measurement, to 3 % for Western blot analysis, to 10 % for –SH groups estimation. These techniques are described below. To exclude a possible osmotic effect of 10 mM Mg^{2+}, the ion was replaced by 10 mM mannitol, not permeating cell membrane and not affecting the parameters studied in the different protocols. Hence, isotonic solution for untreated erythrocytes had the following composition in mM: 140 NaCl, 10 HEPES (4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid), 10 mannitol, pH 7.4, osmotic pressure 305 mOsm. With regard to samples pre-treated with Mg^{2+}, the isotonic solution had the following composition in mM: 150 Choline chloride, 10 KCl, 5 HEPES, 5 glucose, 10 $MgCl_2$, pH 7.4, osmotic pressure 303 mOsm.

$SO_4^=$ uptake measurement

Control conditions. $SO_4^=$ uptake measurement was used to monitor anion exchange capability through Band 3 protein in erythrocytes incubated in a Cl^- free medium, according to what previously described [17, 29], with or without 10 mM Mg^{2+}. Briefly, after washing in isotonic solution, erythrocytes were suspended to 3 % concentration in $SO_4^=$ medium (composition in mM: 118 Na_2SO_4, 20 HEPES, 15 glucose, pH 7.4, osmotic pressure 300 mOsm) and then treated with 10 µM DIDS [30] to block Band 3 protein at specified time intervals.

Trapped $SO_4^=$ was precipitated, spectrophotometrically quantified (425 nm wavelength) and absorption converted to $[SO_4^=]$ L cells x 10^{-2} using a calibrated standard curve obtained by precipitating known $SO_4^=$ concentrations $[SO_4^=]$. L cells x 10^{-2} represents $SO_4^=$ concentration internalized by 10 mL erythrocytes suspended at 3 % concentration.

To verify the efficiency of anion exchange in all experimental conditions, the amount of $SO_4^=$ internalized after 45 min of incubation in $SO_4^=$ medium (once reached equilibrium) was considered, along with the rate constant for $SO_4^=$ uptake.

The rate constant in min^{-1}, calculated according to [29] is reported in Table 1 *per* each experimental condition and accounts for time needed to reach 63 % of total $SO_4^=$ intracellular concentration.

To prove that $SO_4^=$ is internalized through Band 3 protein, the rate constant for $SO_4^=$ uptake was also measured in erythrocytes suspended at 3 % concentration and treated with 10 µM DIDS at the beginning of incubation in $SO_4^=$ medium. Five mL samples were then withdrawn at fixed time intervals and handled as in control conditions.

Table 1. Rate constant (min^{-1}) of $SO_4^=$ uptake. Rate constant (min^{-1}) of $SO_4^=$ uptake measured in untreated erythrocytes, in control conditions (Mg^{2+}-treated erythrocytes) or in erythrocytes treated with either H_2O_2 at different concentrations (300 and 600 µM) or 10 mM Mg^{2+} + either 300 µM H_2O_2 or 600 µM H_2O_2 or treated with either NEM at different concentrations (0.5, 1 or 2 mM) or 10 mM Mg^{2+} + either 0.5 mM, 1 mM or 2 mM NEM. Time (reciprocal of rate constant, min) needed to reach 63% of $SO_4^=$ intracellular concentration saturation is also reported. Data are presented as means ± SD from separate N experiments, where: ***p<0.001 and **p<0.01 versus control; n.s. not significant versus control; §§p<0.01 versus 10 mM Mg^{2+} + 300 µM H_2O_2, §§§p<0.001 versus 10 mM Mg^{2+} + 600 µM H_2O_2 and °°°p<0.001 versus 0.5 mM, 1 mM or 2 mM NEM respectively

Rate constant (min^{-1})		Time (min)	% decrease vs control
Mg^{2+}-untreated erythrocytes	0.055±0.001	18	5
Mg^{2+}-treated erythrocytes (control)	0.058±0.001	17	0
10 µM DIDS	0.018±0.001 ***	55	69
300 µM H_2O_2	0.033±0.001 ***	30	43
600 µM H_2O_2	0.031±0.001 ***	32	47
10 mM Mg^{2+}+300 µM H_2O_2	0.058±0.005 n.s.,§§	17	0
10 mM Mg^{2+}+600 µM H_2O_2	0.057±0.001 n.s.,§§§	17	2
0.5 mM NEM	0.030±0.001 ***	33	48
1 mM NEM	0.033±0.003 ***	30	43
2 mM NEM	0.023±0.002 ***	43	61
10 mM Mg^{2+}+0.5 mM NEM	0.060±0.002 n.s., °°°	16	0
10 mM Mg^{2+}+1 mM NEM	0.056±0.002 n.s., °°°	18	4
10 mM Mg^{2+}+2 mM NEM	0.055±0.002 n.s., °°°	18	6

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Exposure to H_2O_2. After pre-incubation with 10 mM Mg^{2+} (or 10 mM mannitol), whole blood was washed and erythrocytes suspended at 3 % concentration in isotonic medium (containing 10 mM $MgCl_2$ or 10 mM mannitol) plus H_2O_2 (300 µM, 600 µM or 1 mM). After 30 min incubation at 25 °C, samples were centrifuged to remove supernatant and erythrocytes re-suspended to 3 % concentration in $SO_4^=$ medium, containing either 300 µM, 600 µM or 1 mM H_2O_2. $SO_4^=$ uptake was then measured as described for control conditions. Results from these experiments were compared with tests performed with H_2O_2 in the absence of Mg^{2+}.

Exposure to NEM. After pre-incubation with 10 mM Mg^{2+} (or 10 mM mannitol), whole blood samples were washed and erythrocytes suspended at 3 % concentration in isotonic medium (added with 10 mM $MgCl_2$ or 10 mM mannitol) plus NEM (either 0.5 mM, or 1 mM, or 2 mM). After 30 min incubation at 25 °C, samples were centrifuged to remove supernatant and erythrocytes re-suspended to 3 % concentration in $SO_4^=$ medium, containing either 0.5 mM, or 1 mM or 2 mM NEM. $SO_4^=$ uptake was then measured as described for control conditions. Results from these experiments were compared with those deriving from tests performed with NEM in the absence of Mg^{2+}.

Intracellular GSH content measurement

Whole fresh blood samples, treated or not with 10 mM Mg^{2+}, were washed thrice to discard plasma and buffy coat, diluted to 3 % concentration in isotonic medium (added with 10 $MgCl_2$ or 10 mM mannitol) and exposed to H_2O_2 (300 µM, 600 µM or 1 mM) or NEM (0.5 mM or 1 mM or 2 mM) for 30 min at 25 °C. Samples were then centrifuged (2000 g, 5 min at 25 °C) and packed erythrocytes were diluted with isotonic medium to 45 % concentration. Successively, 50 µL NEM (N-ethylmaleimide, from 310 mM stock solution previously dissolved in ethanol pH 7.4) were added. Samples were then centrifuged (2000 g, 5 min at 25 °C), diluted with 1 ml of isotonic medium (1370 g, 10 min, 4 °C) and stored at −80 °C until use. GSH assay [31] is based on the oxidation of GSH by DTNB (5, 5′-dithiobis (2-nitrobenzoic acid)) also called Ellman's reagent, producing GSSG (oxidized glutathione) and TNB (2-nitro-5-thiobenzoic acid), with maximal absorbance at 412 nm. GSH levels were expressed as µM concentration.

Membrane -SH groups levels estimation

Estimation of membrane -SH groups was performed according to Roy et al. [32], with slight modifications, on erythrocytes treated or not with 10 mM Mg^{2+}, washed and diluted to 3 % concentration in isotonic medium (added with 10 mM Mg^{2+} or with 10 mM mannitol). Samples were then exposed for 30 min at 25 °C to, alternatively, H_2O_2 (300 µM, 600 µM or 1 mM) or NEM (0.5 mM or 1 mM or 2 mM). Erythrocytes were successively washed with isotonic medium, diluted to 10 % concentration and lysed by cold hypotonic medium (2.5 mM NaH_2PO_4,). After 10 min stirring at 0 °C, hemoglobin and intracellular content were discarded by repeated centrifugations (4 °C, 18000 g, 20 min). One volume of membranes was then incubated with nine volumes of 0.1 M NaOH for 30 min at 0 °C in presence of 200 µM DTT (dithiothreitol) and 20 µg/ml PMSF (Phenylmethylsulfonil fluoride). After incubation, samples were centrifuged (4 °C, 18000 g, 60 min) and a Band 3 protein-containing pellet was obtained. Membranes were washed thrice with cold hypotonic medium (2.5 mM NaH_2PO_4,) and used for -SH groups determination. In particular, 200 µl pellet were solubilized by incubating 300 µl of 20% w/v SDS (Sodium dodecyl sulphate) reagent in 3 ml of 100 mM sodium phosphate (pH 8.0), for 30 min at 37 °C. Samples were further incubated with 100 µl of 10 mM DTNB (5, 5'-dithiobis-(2-nitrobenzoic acid) in 100 mM sodium phosphate (pH 8.0), for 20 min at 37 °C, which reacts with thiol groups producing a highly colored yellow anion. Levels of -SH groups were then spectrophotometrically detected at 412 nm and –SH groups quantity was expressed as percentage with respect to untreated erythrocytes.

Erythrocytes membranes preparation and SDS-PAGE

Erythrocyte membranes were prepared as previously described [22] with slight modifications. Briefly, after treatment with or without 10 mM Mg^{2+} plus either H_2O_2 or NEM, as reported above, packed erythrocytes were lysed and repeatedly centrifuged (18000 g, 4 °C) until hemoglobin was discarded. Membrane were then solubilized and protein content was measured [33]. Samples were loaded as follows: 2 µg of proteins for anti-Band 3 protein, 30 µg for both anti-Syk and anti-phosphotyrosine (P-Tyr).

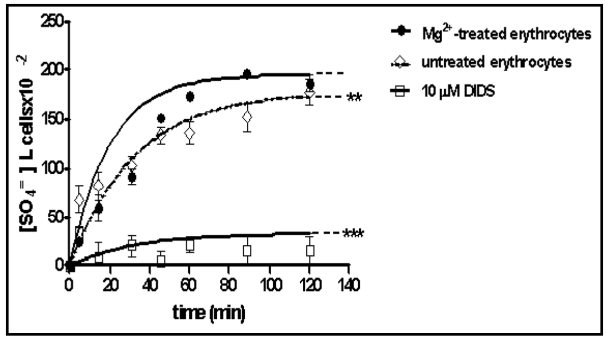

Fig. 1. Time course of $SO_4^=$ uptake in human erythrocytes with or without 10 mM Mg^{2+} or treated with 10 μM DIDS. Points represent the mean ± SD from at least 5 separate experiments (see Table 1), where $^{**}p<0.01$ and $^{***}p<0.001$ versus Mg^{2+}-treated erythrocytes.

Western blot analysis

Western blot analysis was performed according to what previously described [22]. Membranes were incubated at 4 °C overnight with the following primary antibodies: monoclonal anti-Band 3 protein (1:100000; Santa Cruz Biotechnology, produced in mouse), polyclonal anti-Syk (1:1000; Santa Cruz Biotechnology, produced in rabbit) and monoclonal anti-P-Tyr (1:1000; Santa Cruz Biotechnology, produced in mouse). Relative expression of bands for Band 3 protein (approximately 95 kDa), Syk (approximately 75 kDa) and P-Tyr (approximately 100 kDa) were imported to analysis software (Image Quant TL, v2003) and standardized to β-actin levels.

Experimental data and statistics

Data are expressed as arithmetic means ± SD. GraphPad Prism software (version 5.00 for Windows; San Diego, CA) was used. Significant differences between means were tested by one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison *post hoc* test. Statistically significant differences were assumed at $p<0.05$; *N* represents the number of independent experiments.

Table 2. $SO_4^=$ amount ($[SO_4^=]$ L cells $x10^{-2}$) trapped by erythrocytes at 45 min of $SO_4^=$ medium incubation. $SO_4^=$ amount ($[SO_4^=]$ L cells x 10^{-2}) trapped by erythrocytes at 45 min of $SO_4^=$ medium incubation, in control conditions (Mg^{2+}-treated erythrocytes) or in erythrocytes treated with either H_2O_2 at different concentrations (300-600 μM) or 10 μM DIDS or 10 mM Mg^{2+} + either 300 μM H_2O_2 or 600 μM H_2O_2 or treated with either NEM at different concentrations (0.5, 1 or 2 mM) or 10 mM Mg^{2+} + either 0.5 mM, 1 mM or 2 mM NEM. Data are presented as means ± SD from separate N experiments, where: $^{***}p<0.001$, $^{**}p<0.01$ $^{*}p<0.05$ versus control; n.s. not significant versus control; $^{§§}p<0.01$ versus 300 μM H_2O_2, $^{§§§}p<0.001$ versus 600 μM H_2O_2; $^{°°°}p<0.001$ versus 0.5 mM, 1 mM or 2 mM NEM respectively

Treatment	$[SO_4^=]$ L cells $x10^{-2}$
Mg^{2+}-treated erythrocytes	151.12±2.67
untreated erythrocytes	143.55±8.9***
10 μM DIDS	4.75±9 ***
300 μM H_2O_2	133.36±18.2 *
600 μM H_2O_2	130.8±3.45 **
10 mM Mg^{2+}+300 μM H_2O_2	140.75±3.4 $^{n.s.,§§}$
10 mM Mg^{2+}+600 μM H_2O_2	160.45±10.3 $^{n.s.,§§§}$
0.5 mM NEM	133.55±5 **
1 mM NEM	99±10.8 ***
2 mM NEM	31.4±7.5 ***
10 mM Mg^{2+}+0.5 mM NEM	171.5±7.5 $^{n.s., °°°}$
10 mM Mg^{2+}+1 mM NEM	121.4±15 $^{°°°}$
10 mM Mg^{2+}+2 mM NEM	109.15±12.3 $^{°°°}$

Results

Fig. 1 describes the uptake of $SO_4^=$ through Band 3 protein as a function of time in both Mg^{2+}–treated and untreated erythrocytes (Mg^{2+} replaced by mannitol).

The velocity of this process is represented by the rate constant for $SO_4^=$ uptake, reported in Table 1. $SO_4^=$ transport in untreated erythrocytes progressively increased and reached equilibrium in 45 min, while in Mg^{2+}–treated erythrocytes the rate constant was significantly higher than that one measured in untreated cells ($p<0.01$, Table 1).

$SO_4^=$ amount trapped by Mg^{2+}-treated erythrocytes at 45 min of incubation in $SO_4^=$ medium was significantly higher than what measured in untreated erythrocytes ($p<0.001$, Table 2), while in both Mg^{2+}-treated and untreated erythrocytes, $SO_4^=$ amount was significantly higher than that one determined in DIDS-treated erythrocytes. Treatment with 10 μM DIDS, applied at the beginning of incubation in $SO_4^=$ medium, completely blocked $SO_4^=$ uptake, resulting in a

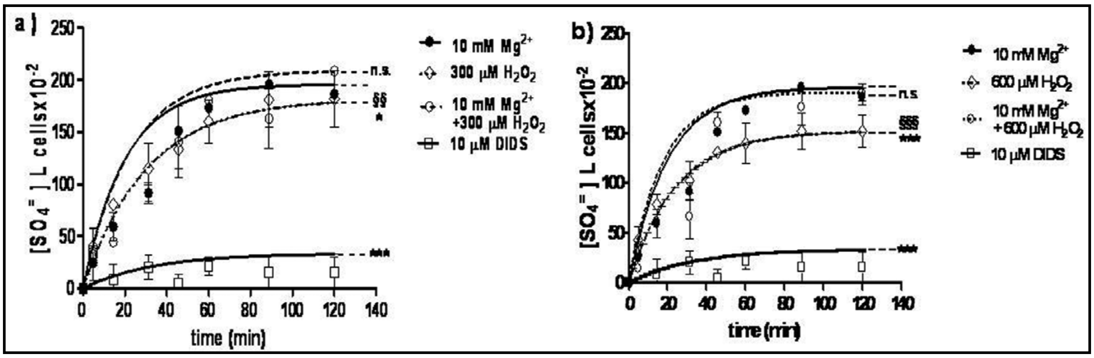

Fig. 2. Time course of $SO_4^=$ uptake in human erythrocytes measured in control conditions (10 mM Mg^{2+}) or in erythrocytes treated with300 μM H_2O_2 or 10 mM Mg^{2+} + 300 μM H_2O_2 (A); or with 600 μM H_2O_2, or 10 mM Mg^{2+} + 600 μM H_2O_2 (B) or 10 μM DIDS (A,B). Points represent the mean ± SD from at least 5 separate experiments (see Table 1), where n.s. is not significant, *p<0.05 and ***p<0.001 versus control; §§p<0.01 versus 300 μM H_2O_2, §§§p<0.001 versus 600 μM H_2O_2.

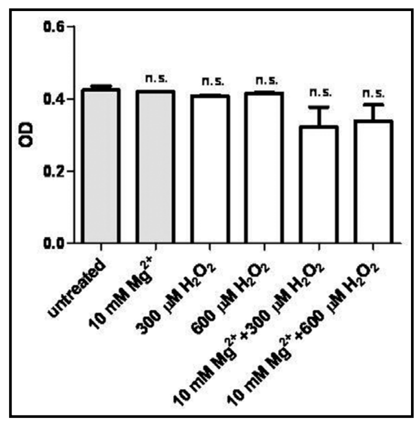

Fig. 3. Intracellular GSH levels measured in untreated erythrocytes, or in 10 mM Mg^{2+}-treated erythrocytes (control) or in H_2O_2-treated (300 μM, 600 μM or 1mM) erythrocytes, with or without pre-exposure to 10 mM Mg^{2+} (10 mM Mg^{2+}+ 300 μM H_2O_2 or 10 mM Mg^{2+}+ 600 μM H_2O_2 or 10 mM Mg^{2+}+ 1 mM H_2O_2). Bars represent the mean ± SD from at least 5 experiments, where 10 mM Mg^{2+} n.s. versus untreated erythrocytes; n.s. versus control.

rate constant significantly lower than that one observed in both Mg^{2+}-treated and untreated erythrocytes (p<0.001, Table 1). Curve related to Mg^{2+}-treated erythrocytes has been assumed as control for $SO_4^=$ uptake experiments under both H_2O_2 and NEM treatment.

H_2O_2 treatment

$SO_4^=$ uptake measurement. Fig. 2A-B describes $SO_4^=$ uptake as a function of time under either 300 μM (A) or 600 μM H_2O_2 (B) with or without pre-exposure to 10 mM Mg^{2+}, and compared to Mg^{2+}-treated erythrocytes, assumed as control. As hemolysis has been detected after incubation in $SO_4^=$ medium plus 1 mM H_2O_2, data have been not considered.

The rate constant for $SO_4^=$ uptake in H_2O_2-treated cells, at both concentrations, was significantly lower than that one measured in control conditions (Table 1), whereas, when Mg^{2+} was applied before H_2O_2 treatment, the rate constant for $SO_4^=$ uptake was brought back

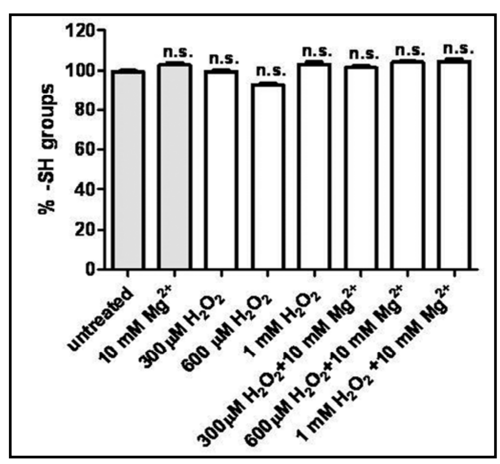

Fig. 4. Percentage of membrane –SH groups measured in untreated erythrocytes, or Mg^{2+}-treated (control) or treated with either 300 μM or 600 μM or 1 mM H_2O_2 with or without 10 mM Mg^{2+} (10 mM Mg^{2+}+ 300 μM H_2O_2 or 10 mM Mg^{2+}+ 600 μM H_2O_2 or 10 mM Mg^{2+}+ 1 mM H_2O_2). Bars represent the mean ± SD from at least 5 experiments, where 10 mM Mg^{2+} n.s. versus untreated erythrocytes; n.s. versus control.

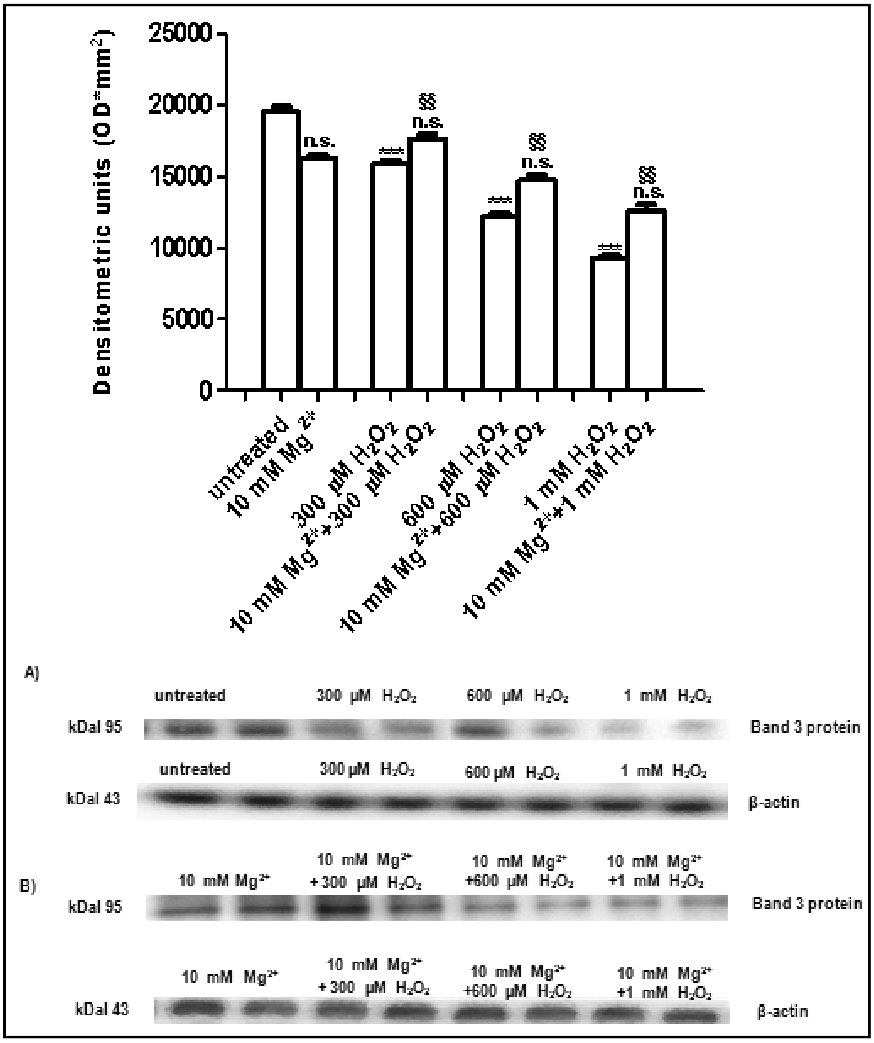

Fig. 5. Expression levels of Band 3 protein measured in untreated erythrocytes, or in Mg^{2+}-treated erythrocytes (control) or in erythrocytes treated with either 300 μM, or 600 μM or 1 mM H_2O_2 (A), or treated with 10 mM Mg^{2+} + either 300 μM, or 600 μM or 1 mM H_2O_2 (B) detected by Western blot analysis. 10 mM Mg^{2+} n.s. versus untreated erythrocytes; n.s. not significant versus control and ***$p<0.001$ versus control; $^{§§}p<0.01$ versus either 300 μM, or 600 μM or 1 mM H_2O_2 alone.

to control values (Table 1). $SO_4^=$ amount trapped at 45 min of $SO_4^=$ medium incubation by erythrocytes exposed to either 300 μM or 600 μM H_2O_2 was significantly lower than that one measured in control erythrocytes (Table 2), while, after exposure to Mg^{2+} plus either 300 μM or 600 μM H_2O_2, it was significantly higher than what measured in, respectively, 300 μM and 600 μM H_2O_2-treated erythrocytes (Table 2). Both $SO_4^=$ amount and the rate constant for $SO_4^=$ uptake in control and experimental conditions were significantly different with respect to the same parameters measured in DIDS-treated cells (Fig. 2A-B, Tables 1-2).

Intracellular GSH content determination. GSH content in untreated erythrocytes was not significantly different with respect to that one measured in Mg^{2+}-treated cells, which has been assumed as control (Fig. 3). Moreover, no significant difference was detected between intracellular GSH concentration in erythrocytes treated with either 300 μM or 600 μM H_2O_2, with or without 10 mM Mg^{2+} and that one measured in control.

Membrane -SH groups determination. Determination of membrane -SH groups, reported as percentage in Fig. 4, was performed in erythrocytes treated with either 300 μM, or 600 μM H_2O_2 or 1 mM H_2O_2, with or without pre-exposure to 10 mM Mg^{2+} and compared to control (Mg^{2+}-treated erythrocytes). Membrane -SH groups have been also estimated in untreated erythrocytes, exhibiting no significant difference with respect to –SH groups levels measured in Mg^{2+}-treated cells. After exposure to either 300 μM, or 600 μM H_2O_2 or 1 mM H_2O_2, membrane -SH groups levels were not significantly different with respect to control, both in presence and in absence of pre-exposure to 10 mM Mg^{2+}.

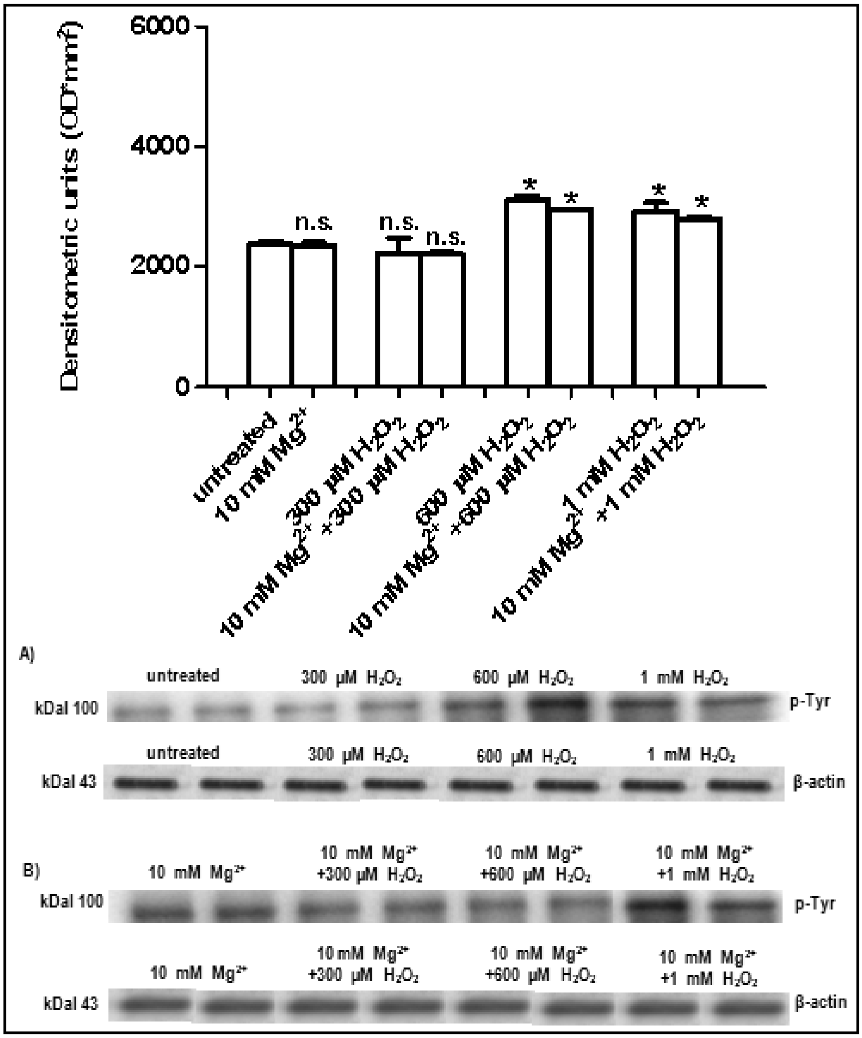

Fig. 6. Expression levels of phosphorylated Tyrosine (P-Tyr) measured in untreated erythrocytes or in Mg^{2+}-treated erythrocytes (control) or in erythrocytes treated with either 300 µM, or 600 µM or 1 mM H_2O_2 (A), or with 10 mM Mg^{2+} + either 300 µM, or 600 µM H_2O_2 or 1 mM H_2O_2 (B), detected by Western blot analysis. 10 mM Mg^{2+} n.s. not significant versus untreated erythrocytes; 300 µM H_2O_2, 10 mM Mg^{2+} + 300 µM H_2O_2 n.s. versus control; 10 mM Mg^{2+} + 600 µM H_2O_2 and 10 mM Mg^{2+} + 1 mM H_2O_2 n.s. not significant versus 600 µM H_2O_2 and 1 mM H_2O_2 respectively; * $p<0.05$ versus control.

Western blot analysis. Fig. 5 shows that Band 3 protein levels in untreated erythrocytes were not significantly different with respect to Mg^{2+}-treated erythrocytes (control), while, after treatment with 300 µM, 600 µM H_2O_2 or 1 mM H_2O_2, they were significantly lower than control ($p<0.001$). Such reduction was reversed by Mg^{2+}-treatment, as a significant difference was detected between Band 3 protein expression levels in erythrocytes treated with H_2O_2 alone (at all concentrations) and those treated with Mg^{2+} plus H_2O_2 ($p<0.01$).

With regard to P-Tyr expression levels, Fig. 6 shows that in untreated erythrocytes they were not significantly different with respect to Mg^{2+}-treated erythrocytes (control). Moreover, P-Tyr expression levels after treatment with 300 µM H_2O_2 were not significantly different with respect to control, while in both 600 µM and 1 mM H_2O_2-treated erythrocytes they were significantly higher than those determined in control ($p<0.05$). P-Tyr expression levels in erythrocytes treated with Mg^{2+} plus H_2O_2 (both 600 µM and 1 mM) were not restored, since a significant difference between P-Tyr expression levels measured in these experimental conditions and those measured in control was seen.

Fig. 7 shows that Syk expression levels in untreated erythrocytes were not significantly different with respect to those measured in Mg^{2+}-treated erythrocytes (control). Syk expression levels, after treatment with either 300 µM, or 600 µM or 1 mM H_2O_2, were significantly higher than those determined in control ($p<0.01$), while, in Mg^{2+} plus H_2O_2-

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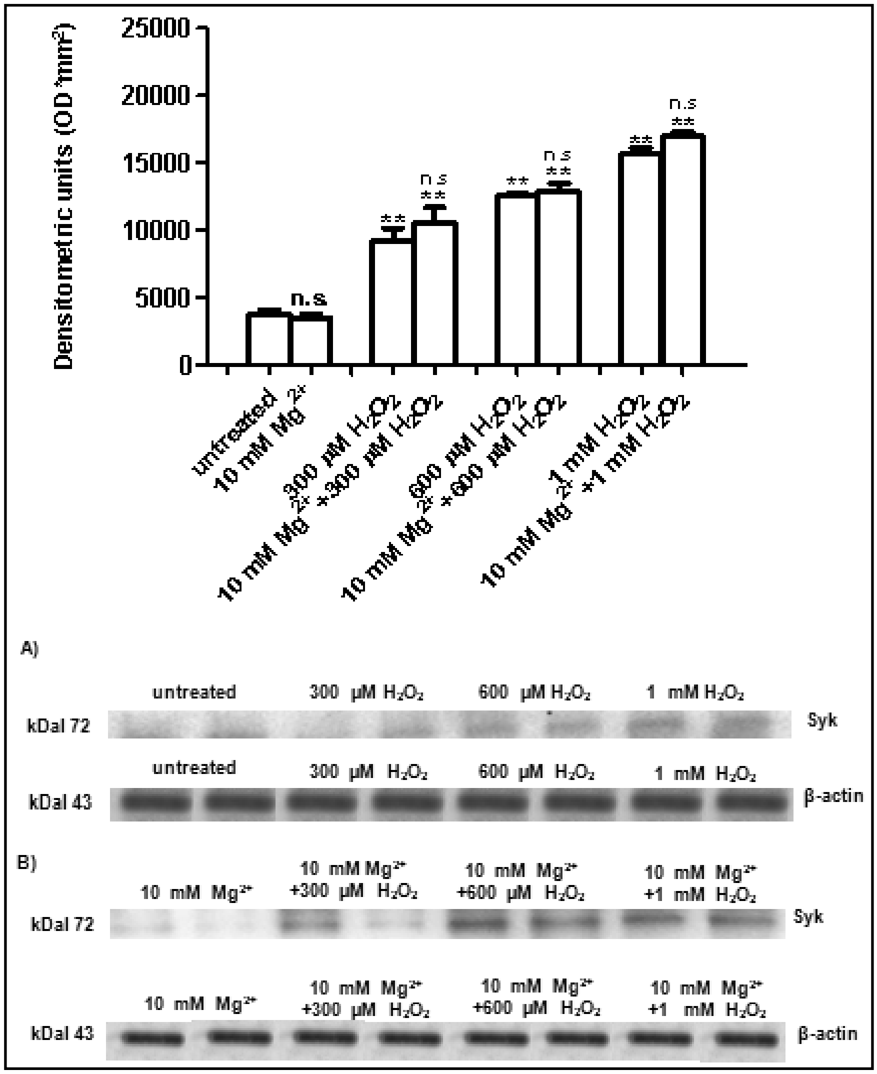

Fig. 7. Expression levels of tyrosine kinase (Syk) measured in untreated erythrocytes or in Mg^{2+}-treated erythrocytes (control) or in erythrocytes treated with either 300 μM, or 600 μM or 1 mM H_2O_2 (A), or with 10 mM Mg^{2+} + either 300 μM, or 600 μM or 1 mM H_2O_2 treatment (B), detected by Western blot analysis. 10 mM Mg^{2+} n.s. not significant versus untreated erythrocytes; **$p<0.01$ versus control, 10 mM Mg^{2+} + either 300 μM, or 600 μM or 1 mM H_2O_2 n.s. not significant versus 300 μM, or 600 μM or 1 mM H_2O_2.

treated erythrocytes (at any concentration), they were not significantly different with respect to those measured under H_2O_2 alone and higher than control ($p<0.01$).

NEM treatment

$SO_4^=$ uptake measurement. Fig. 8 shows the time course for $SO_4^=$ uptake in either NEM-treated erythrocytes (A: 0.5 mM, B: 1 mM, C: 2 mM) or in Mg^{2+}-treated erythrocytes (control).

$SO_4^=$ amount trapped at 45 min of incubation in $SO_4^=$ medium by erythrocytes exposed to NEM (at any concentration) was significantly lower (Table 2) than $SO_4^=$ internalized by control erythrocytes, while, after exposure to 10 mM Mg^{2+} plus either 0.5 mM, or 1 mM or 2 mM NEM, it was significantly higher than that one measured in NEM-treated erythrocytes (at any concentration) (Table 2). The rate constant for $SO_4^=$ uptake in NEM-treated cells at 0.5 mM, 1 mM and 2 mM was significantly lower than that one measured in control conditions (Table 1). When Mg^{2+} was applied before NEM at all concentrations, the rate constant for $SO_4^=$ uptake was significantly improved (Table 1). Both $SO_4^=$ amount and rate constant for $SO_4^=$ uptake in control and experimental conditions were significantly different with respect to the same parameters measured in DIDS-treated cells (Fig. 8A-C, Tables 1-2).

Intracellular GSH content determination. GSH levels in erythrocytes treated with either 0.5 mM, or 1 mM, or 2 mM NEM, with or without 10 mM Mg^{2+}, are reported in Fig. 9. GSH levels in Mg^{2+}-treated cells (control) were not significantly different with respect to those

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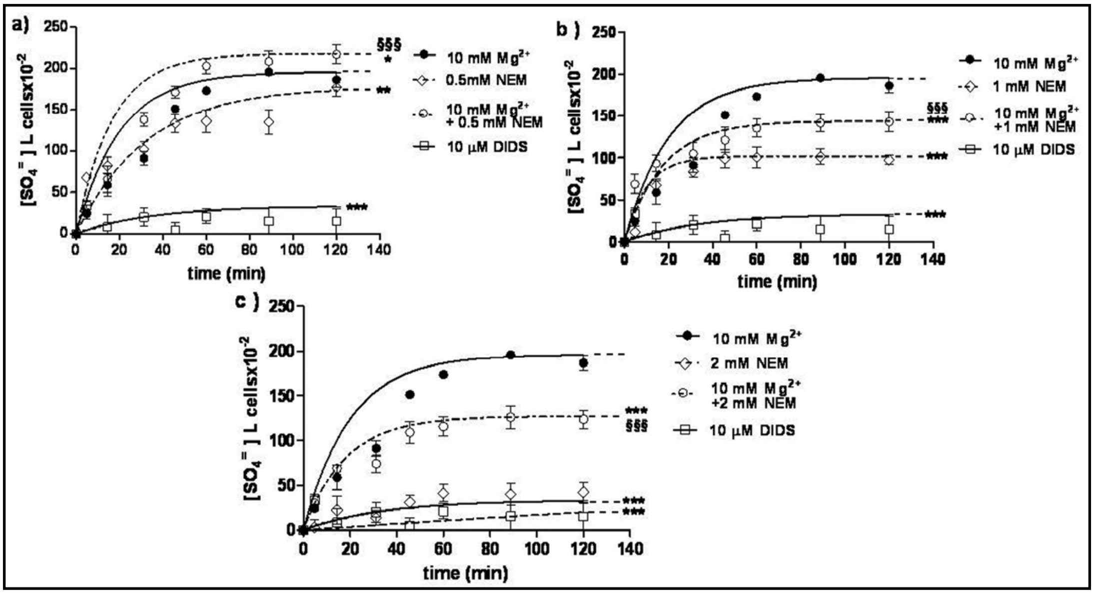

Fig. 8. Time course of $SO_4^=$ uptake in human erythrocytes measured in control conditions (10 mM Mg^{2+}) or with 0.5 mM (A), or 1 mM (B) or 2 mM NEM (C), or with 10 mM Mg^{2+} + either 0.5 mM (A), or 1 mM (B) or 2 mM NEM (C), or with 10 μM DIDS (A-B-C). Points represent the mean ± SD from at least 5 separate experiments (see Table 1), where ***$p<0.001$, **$p<0.01$ and *$p<0.05$ versus control; °°°$p<0.001$ versus either 0.5 mM, or 1 mM, or 2 mM NEM.

determined in untreated erythrocytes. After exposure to either 0.5 mM, or 1 mM, or 2 mM NEM, GSH levels were significantly lower than control ($p<0.001$). In erythrocytes treated with 10 mM Mg^{2+} + either 0.5 mM, or 1 mM, or 2 mM NEM, GSH levels were not significantly different with respect to those of control, while significantly higher than those measured after treatment with NEM alone at any concentration ($p<0.001$).

Membrane-SH groups determination. Membrane -SH groups determination, as depicted in Fig. 10, was performed after exposure to different concentrations of NEM (0.5 mM, or 1 mM or 2 mM) with or without 10 mM Mg^{2+}. Levels of membrane -SH groups in Mg^{2+}-treated cells (control) were not significantly different with respect to those determined in untreated erythrocytes. Levels of -SH groups after treatment with NEM were significantly lower than those of control ($p<0.001$), while, in erythrocytes pre-treated with 10 mM Mg^{2+} before NEM (0.5 mM, 1 mM or 2 mM), they were significantly higher than those measured under NEM treatment alone ($p<0.05$, $p<0.001$) and significantly lower than those measured in control ($p<0.05$, $p<0.001$).

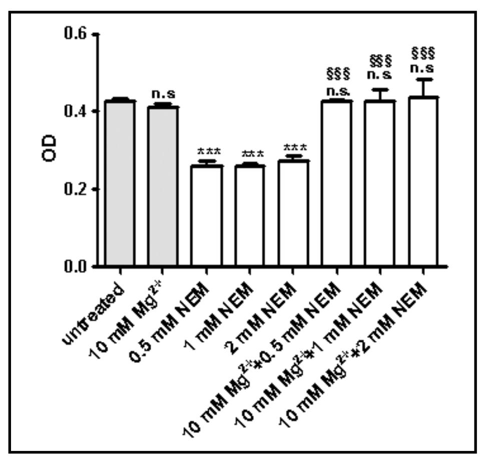

Fig. 9. Intracellular GSH levels (μM) measured in untreated erythrocytes or in Mg^{2+}-treated erythrocytes (control), or in erythrocytes treated with 0.5 mM, or 1 mM, or 2 mM NEM with or without 10 mM Mg^{2+}(10 mM Mg^{2+}+ 0.5 mM NEM or 10 mM Mg^{2+}+ 1 mM NEM or 10 mM Mg^{2+} + 2 mM NEM). Bars represent the mean ± SD from at least 5 experiments, 10 mM Mg^{2+} n.s. not significant versus untreated; n.s. not significant versus control, ***$p<0.001$ versus control; °°°$p<0.001$ versus either 0.5 mM, or 1 mM, or 2 mM NEM.

Western blot analysis. Band 3 protein expression levels in untreated erythrocytes, as depicted in Fig. 11, were not significantly different with respect to those measured in Mg^{2+}-treated erythrocytes (control). After treatment with either 0.5 mM, or 1 mM, or 2 mM NEM, they were significantly lower than control ($p<0.001$), while, in erythrocytes pre-treated with 10 mM Mg^{2+} before NEM (0.5 mM, or 1 mM or 2 mM), they were higher than those measured under NEM treatment alone ($p<0.001$ and $p<0.01$), and not significantly different with respect to control.

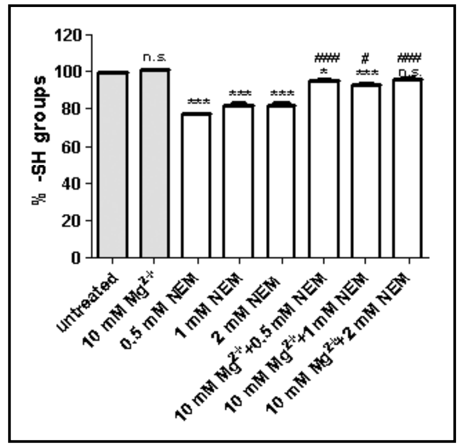

Fig. 10. Percentage of membrane –SH groups measured in untreated erythrocytes, or in 10 mM Mg^{2+}-treated erythrocytes (control), or in erythrocytes treated with 0.5 mM, or 1 mM, or 2 mM NEM with or without 10 mM Mg^{2+} (10 mM Mg^{2+}+ 0.5 mM NEM or 10 mM Mg^{2+}+ 1 mM NEM or 10 mM Mg^{2+}+ 2 mM NEM). Bars represent the mean ± SD from at least 5 experiments. 10 mM Mg^{2+} n.s. not significant versus untreated, ***$p<0.001$ versus control; 10 mM Mg^{2+} + 2 mM NEM n.s. not significant versus control, °$p< 0.05$ versus 1 mM NEM, °°°$p<0.001$ versus 0.5 mM and 2 mM NEM.

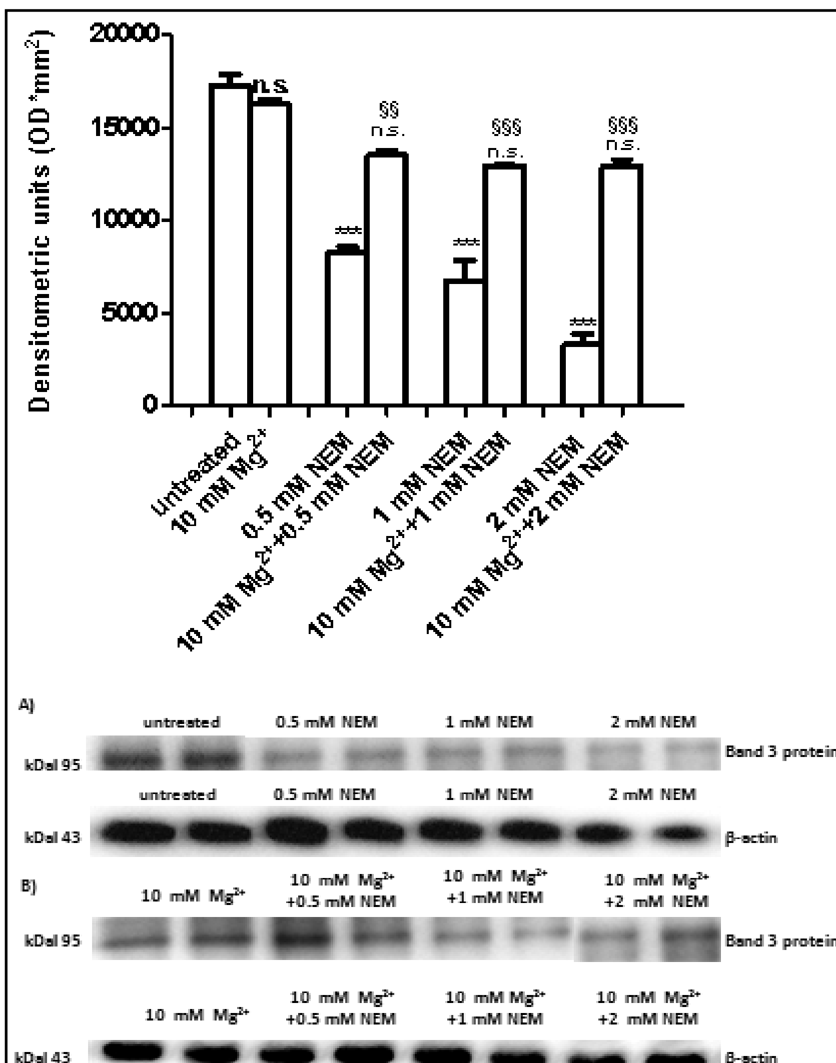

Fig. 11. Expression levels of Band 3 protein measured in untreated erythrocytes, or in 10 mM Mg^{2+}-treated erythrocytes (control), or in erythrocytes treated with either 0.5 mM, or 1 mM or 2 mM NEM (A) and with 10 mM Mg^{2+} + either 0.5 mM, or 1 mM or 2 mM NEM (B), detected by Western blot analysis. 10 mM Mg^{2+} n.s. not significant versus untreated,***$p<0.001$ versus control, 10 mM Mg^{2+} + either 1 mM or 2 mM NEM °°°$p<0.001$ versus 1 mM or 2 mM NEM, 10 mM Mg^{2+} + 0.5 mM °°$p<0.01$ versus 0.5 mM NEM, 10 mM Mg^{2+} + either 0.5 mM, or 1 mM or 2 mM NEM n.s. not significant versus control.

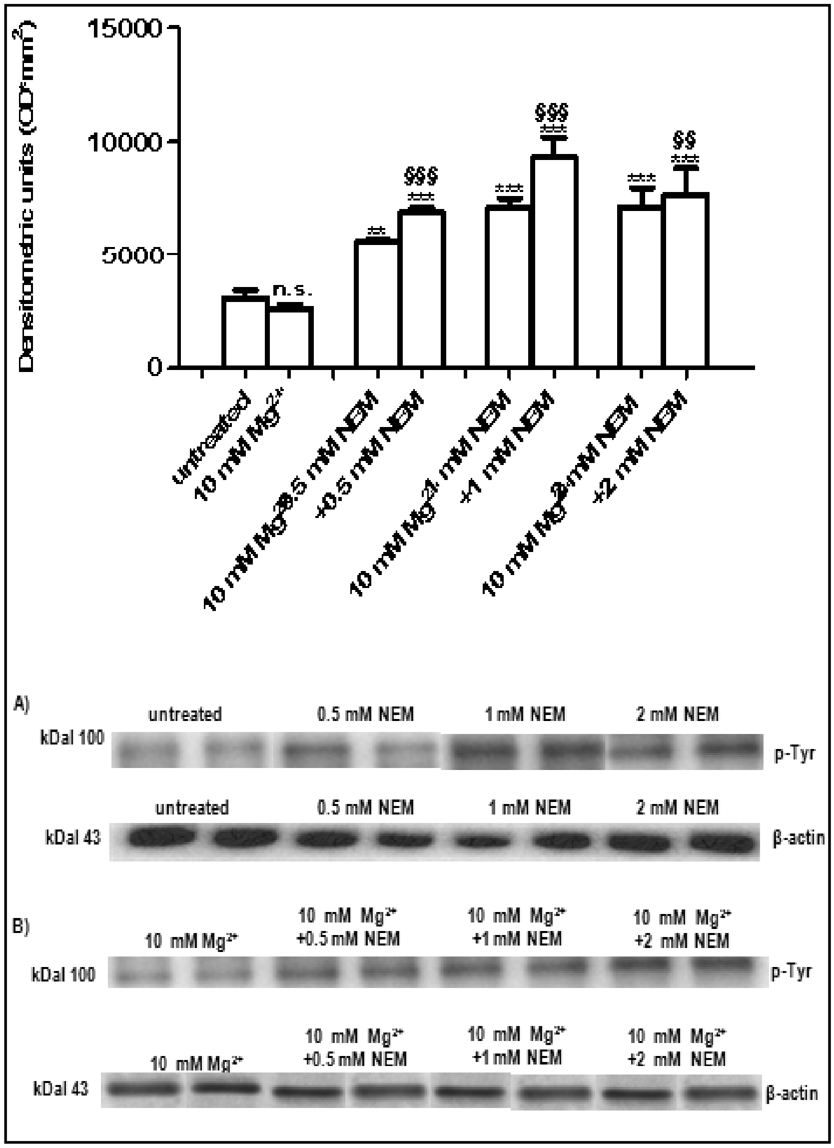

Fig. 12. Expression levels of phosphorylated Tyrosine (P-Tyr) measured in untreated erythrocytes, or in 10 mM Mg^{2+}-treated erythrocytes (control) or in erythrocytes treated with either 0.5 mM, or 1 mM, or 2 mM NEM (A) and treated with 10 mM Mg^{2+} + either 0.5 mM, or 1 mM, or 2 mM NEM (B), detected by Western blot analysis. 10 mM Mg^{2+} n.s. not significant versus untreated; ***p<0.001 and **p<0.01 versus control.

P-Tyr expression levels in untreated erythrocytes, as shown in Fig. 12, were not significantly different with respect to those measured in Mg^{2+}-treated erythrocytes (control). P-Tyr expression levels, after treatment with either 0.5 mM, or 1 mM or 2 mM NEM, were significantly higher than those of control ($p<0.01$ and $p<0.001$). When Mg^{2+} was applied before NEM treatment, P-Tyr expression levels were significantly higher than control ($p<0.01$ and $p<0.001$).

Fig. 13 shows that Syk expression levels in untreated erythrocytes were not significantly different with respect to those measured in Mg^{2+}-treated erythrocytes (control). After treatment with either 0.5 mM, or 1 mM, or 2 mM NEM, Syk expression levels were not significantly different with respect to those of control. Moreover, levels measured in Mg^{2+} plus NEM-treated erythrocytes, at all concentrations, were not significantly different with respect to those determined under NEM treatment alone and with respect to control.

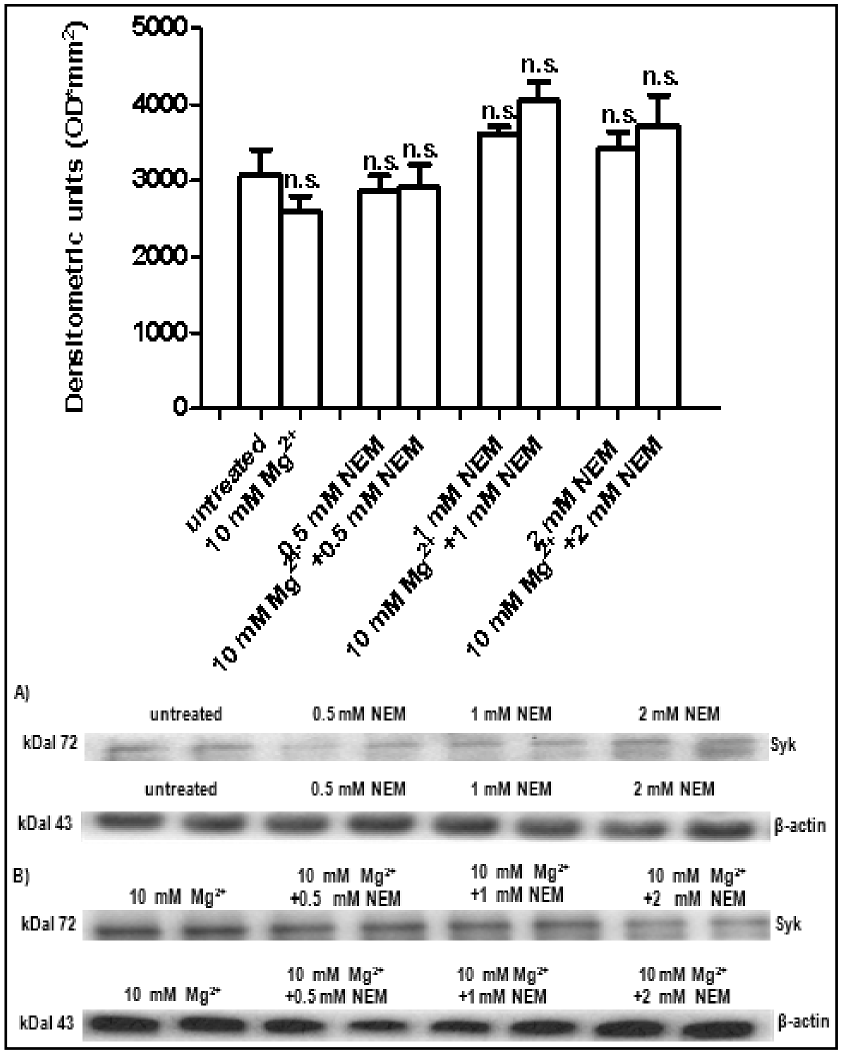

Fig. 13. Expression levels of tyrosine kinase (Syk) measured in untreated erythrocytes, or in 10 mM Mg^{2+}-treated erythrocytes (control) or in erythrocytes treated with either 0.5 mM, or 1 mM, or 2 mM NEM (A) and treated with 10 mM Mg^{2+} + either 0.5 mM, or 1 mM, or 2 mM NEM treatment (B), detected by Western blot analysis. 10 mM Mg^{2+} n.s. not significant versus untreated; n.s. not significant versus control.

Discussion

The beneficial effect of Mg^{2+} supplementation has been already proven in oxidative stress-related diseases, such as preeclampsia and hypoxia due to preterm labour [5, 34]. Amongst diseases associated to oxidative stress, systemic sclerodermia, diabetes, β-thalassemia and canine leishmaniasis may result in an altered anion exchange capability through Band 3 protein [25-28], which is essential to erythrocytes homeostasis and oxygenation of whole organism [11, 12]. On these premises and with the purpose of clarifying the beneficial effect of Mg^{2+} supplementation in case of diseases related to oxidative conditions, the present investigation aimed to verify whether Band 3 protein and underlying phosphorylative pathways are a target for a possible Mg^{2+} antioxidant action. To this end, two different *in vitro* models of oxidative stress have been used.

The present findings confirm that the oxidants chosen to set up the experiments, H_2O_2 and NEM, significantly reduce the rate constant for $SO_4^=$ uptake through Band 3 protein, previously validated as a suitable tool accounting for erythrocytes homeostasis [29].

H_2O_2 reduces the rate constant for $SO_4^=$ uptake by either formation of hemoglobin aggregates, altering binding with Band 3 protein and cross-link with cytoskeletal proteins [7, 20, 35], or by protein degradation [36], while, on the other hand, NEM inhibits $SO_4^=$ uptake through –SH membrane groups and intracellular GSH oxidation [6], Here we show that pre-exposure to Mg^{2+} impairs the reduction in anion exchange capability after oxidation with both molecules, raising thus the question about Mg^{2+} mechanism of action.

As Mg^{2+} has been already demonstrated to slightly accelerate the rate constant for $SO_4^{=}$ uptake in the absence of oxidative conditions [5, 6], the hypothesis is that its effect could compensate the reduction induced by an exposure to H_2O_2, which is also in line with Crupi et al. [26], showing that Mg^{2+} accelerates the rate of anion exchange in erythrocytes from β-thalassemic patients, a disease associated to oxidative conditions. The use of high H_2O_2 concentrations (1 mM) let us demonstrate that neither intracellular GSH levels nor membrane –SH groups are affected by oxidant conditions and that Mg^{2+} may protect membrane structure. The evidence that natural antioxidants may preserve erythrocytes membrane from oxidative damage has been already provided [17]. In any case, lower H_2O_2 concentrations (not hemolytic) are sufficient to prove alterations of Band 3 protein function and, in addition, are useful to prove Mg^{2+} beneficial effect.

However, since Mg^{2+} impairs oxidative damage on –SH groups of membrane proteins (mostly belonging to Band 3 protein [32]) and on intracellular GSH, as shown by NEM experiments, the hypothesis of an effect of this metal at endogenous antioxidant system level can't be excluded, in line with what previously described by our group [22].

At this point, we may conclude that Mg^{2+} protects erythrocytes homeostasis *via* two different mechanisms, one dealing with membrane organization and cross link between proteins and the other one dealing with intracellular GSH levels maintenance.

As a further step, Band 3 protein expression levels have been measured. Here we show for the first time that Mg^{2+} pre-exposure prevents Band 3 protein expression levels reduction provoked by both oxidant molecules. To explain this result, an increase in the number of Band 3 proteins on erythrocytes membrane under Mg^{2+} treatment may be suggested, as Chernyshova et al. [5] did. Nevertheless, as these authors proved a Mg^{2+}-dependent increased number of Band 3 protein due to an augmented number of erythrocytes *in vivo* and, of course, being synthesis of new Band 3 proteins in anucleated cells excluded [22], such hypothesis seems not to be applicable to the present *in vitro* model.

Therefore, Mg^{2+} would more likely impair membrane protein degradation/aggregation which means protection of membrane flexibility. As said above, Band 3 protein aggregation/degradation is provoked by oxidative stress and is responsible for a decrease in Band 3 protein expression levels [35-36]. In particular, these authors report about modified forms of hemoglobin (hemicromes) binding to erythrocytes membrane and resulting in increased Band 3 protein degradation products, namely on senescent erythrocytes. Hence, the present evidence that Mg^{2+} impairs a reduction in Band 3 protein expression levels under oxidant conditions would suggest Mg^{2+} as a good candidate, i.e. by food supplementation, to preserve membrane flexibility and, in turn, rheological properties of blood, crucial features in oxidative stress-related pathologies.

As a further step, the signaling modulating Band 3 protein efficiency has been for the first time considered to prove the beneficial effect of Mg^{2+} under oxidative stress. As previous investigations showed a correlation between oxidative stress, high levels of P-Tyr and kinase role [34, 37], our purpose was to determine expression levels of P-Tyr along with those of Syk kinase. In this regard, Zipser et al. [37] have already demonstrated that Mg^{2+} may activate dephosphorilation of Band 3 protein when blocked by Ca^{2+}.

Band 3 protein has been described as a substrate of Ser/Thr kinases and major substrate of tyrosine kinases [38], specifically Lyn and Syk. Syk kinase is responsible for phosphorylation of tyrosines 8 and 21, associated to Band 3 protein oxidation [39] producing a binding site for other protein tyrosine kinases [18, 39]. Tyrosine (Tyr) phosphorylation in erythrocytes [37, 40] reflects the balance between the competing activities of protein tyrosine kinases and protein tyrosine phosphatases (PTPs) [18], whose activity mostly contributes to keep protein phosphotyrosine (P-Tyr) basal levels very low [41, 42].

According to the present results, neither Syk upregulation nor P-Tyr upregulation, observed after both 600 μM and 1 mM H_2O_2 treatment, are impaired by pre-exposure to Mg^{2+}. However, high levels of Syk may not always correspond to high levels of P-Tyr, as shown under

300 µM H_2O_2 treatment. This observation is in agreement with Pantaleo et al. [43], reporting that such discrepancy may depend on oxidized Band 3 protein molecules impairing Syk docking, which makes thus Syk ineffective in phosphorylating Tyr residues. Therefore, we may state that Mg^{2+} beneficial effect is not mediated by phosphorylative pathways, but would rather prevent alterations in cross link between Band 3 and cytoplasmatic proteins [7], impairing Syk docking. In this regard, the evidence that Mg^{2+} promotes Band 3 protein anion exchange capability in intact erythrocytes but not in ghosts (resealed erythrocytes deprived of cytoplasmic content) has been already provided [7]. On the other hand, an involvement of a kinase different from Syk may be also suggested [37].

At higher H_2O_2 concentrations, the improved anion exchange capability under Mg^{2+} pre-treatment would depend on the stabilization of Hb/Band 3 protein binding, compensating the reduction possibly due to high phosphorylation levels.

Many authors have demonstrated that Tyr phosphorylation can be increased by compounds known to inhibit PTPs, normally acting to maintain very low P-Tyr levels [41], and NEM is one of them [44].

Upon the present data, Syk seems not to mediate Tyr phosphorylation increased by NEM treatment [45], suggesting that other protein kinases, such as Lyn [18], may be involved. Moreover, high P-Tyr levels, as already said, may also derive from a NEM-induced inhibition of PTP, not restored by Mg^{2+} treatment

Even though Mg^{2+} has been shown to promote phosphorylative events in case of deoxygenation [47], this would not be the case, as no difference in P-Tyr levels has been detected between NEM-treated and Mg^{2+} plus NEM-treated erythrocytes. Therefore, the rate constant restoration due to Mg^{2+} pre-exposure seems not to depend on phosphorylative pathways modulation, but rather on GSH and –SH groups protection, suggesting a role of endogenous antioxidant system stabilized by Mg^{2+}.

Conclusion

In conclusion, the use of H_2O_2 and NEM to model oxidant conditions on erythrocytes allows to prove that the anion exchange capability through Band 3 protein is a useful tool to detect the beneficial effects of Mg^{2+} against oxidative stress in cells continuously exposed to free radicals. Such beneficial effect is putatively mediated by erythrocytes endogenous antioxidant system activity, in addition to a stabilization of the crosslink between Band 3 protein and cytoplasmic proteins.

The present study, comprised in a wider investigation dealing with the use of natural antioxidants to preserve erythrocytes homeostasis from oxidative conditions, confirms Band 3 protein as a target for beneficial effect action Mg^{2+} and may support the use of Mg^{2+}-containing food supplements in case of oxidative stress-related diseases possibly affecting anion exchange capability, such as Systemic sclerodermia, diabetes, hypertension and canine leishmaniasis [25-28].

Acknowledgements

R.M. conceived and performed the experiments, analysed data; A.R. performed experiments and analysed data; A.M. conceived the experiments, wrote and revised the manuscript.

Disclosure Statement

The authors have no conflicts of interest to declare.

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