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Glutamine protects intestinal calcium absorption against oxidative stress and apoptosis

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ABSTRACT

The aim of this study was to investigate whether glutamine (GLN) could block the inhibition of the intestinal Ca^{2+} absorption caused by menadione (MEN), and elucidate the underlying mechanisms. To do this, one-month old chicks were divided in four groups: 1) controls, 2) MEN treated, 3) GLN treated and 4) GLN treated before or after MEN treatment. Intestinal Ca^{2+} absorption as well as protein expression of molecules involved in the transcellular Ca^{2+} pathway were determined. Glutathione (GSH) and superoxide anion and activity of enzymes of the antioxidant system were evaluated. Apoptosis was measured by the TUNEL technique, the expression of FAS and FASL and the caspase-3 activity. A previous dose of 0.5 g GLN/kg of b.w. was necessary to show its protector effect and a dose of 1 g/kg of b.w. could restore the intestinal Ca^{2+} absorption after MEN treatment. GLN alone did not modify the protein expression of calbindin D_{28k} and plasma membrane Ca^{2+}-ATPase, but blocked the inhibitory effect of the quinone. GLN avoided changes in the intestinal redox state provoked by MEN such as a decrease in the GSH content, and increases in the superoxide anion and in the SOD and CAT activities. GLN abrogated apoptotic effects caused by MEN in intestinal mucosa, as indicated by the reduction of TUNEL (+) cells and the FAS/FASL/caspase-3 pathway. In conclusion, GLN could be an oral nutritional supplement to normalize the redox state and the proliferation/cell death ratio in the small intestine improving the intestinal Ca^{2+} absorption altered by oxidative stress.

1. Introduction

Glutamine (GLN) is classified as a non essential aminoacid, but it appears essential for the viability and growth of intestinal cells. It is the primary metabolic fuel of enterocytes from the small intestine, where it is a precursor in nucleotide, glucose and amino sugar, and protein synthesis (Wang et al., 2015). GLN metabolism by the intestinal epithelium generates substantial quantities of glutamate, a component for glutathione synthesis which is the main molecule responsible for the maintenance of the cellular redox state (Alves et al., 2010; Newsholme et al., 2003). GLN administration was capable of preserving the intestinal barrier in mice intestinal obstruction reducing the intestinal permeability and the bacterial translocation (Dos Santos et al., 2010). It has also been observed that oral GLN ameliorates chemotherapy-induced changes of intestinal permeability without interfering with the antitumor effect of chemotherapy in patients with breast cancer (Li et al., 2006). Shu et al. (2014) have shown that GLN reduces the endotoxin translocation, decreases inflammation and improves immune barrier function, which suggests that GLN would also improve the outcomes of liver transplantation. It has also been demonstrated that GLN has protective effects on ischemia/reperfusion (I/R) *in vivo* by activating the Nrf2/ARE signaling pathway to inhibit ROS production and decrease intestinal apoptosis (Wang et al., 2015), as well as by downregulating the high mobility group box 1 protein and the inflammatory cytokine expression (Shu et al., 2016). In other words, GLN is not only essential for the enterocytes as a fuel or precursor of main

Abbreviations: GLN, glutamine; MEN, menadione; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase; CB D_{28k}, calbindin D_{28k}; $PMCA_{1b}$, plasma membrane Ca^{2+}-ATPase; I/R, ischemia/reperfusion; ROS, reactive oxygen species; OD, optical density; DAB, 3,3′-diaminobenzidine; $\cdot O_2^-$, superoxide anion; NBT, nitro blue tetrazolium; TUNEL, terminal transferase-mediated dUTP nick-end labeling procedure.; HSP, heat shock protein; ERK, extracellular signal-regulated kinases; MAPK, Mitogen-activated protein kinases; TGF-β1, Transforming growth factor beta 1; Smad3, Mothers against decapentaplegic homolog 3

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components, but also for of its antioxidant, antiapoptotic and antiinflammatory properties.

The intestine is the only gate for the Ca^{2+} entrance into the organism. This physiological process occurs through paracellular and transcellular pathways (Diaz de Barboza et al., 2015) and depends on the ATP hydrolysis and the intestinal GSH content (Marchionatti et al., 2008), among other factors. The tripeptide depletion caused by menadione (MEN) or vitamin K_3 produces inhibition of intestinal Ca^{2+} absorption (Marchionatti et al., 2013). Supplementation of antioxidants such as quercetin and melatonin can prevent or block the inhibitory effect of MEN. Since GLN has antioxidant and antiapoptotic properties and has the advantage of being an oral nutritional supplement (Chaudhry et al., 2016), it could be used to prevent or restore the inhibition of the intestinal Ca^{2+} absorption produced by MEN, other GSH depleting drugs or conditions such as aging (Pascua et al., 2011), inflammatory bowel disease (Almenier et al., 2012), celiac disease (Ferretti et al., 2012), gut cancer (Circu and Aw, 2011) that occur with an enhancement of ROS and reactive nitrogen species, which in turn would cause alteration in the intestinal Ca^{2+} absorption.

Based upon previous considerations, the purpose of the present study is to investigate whether GLN could prevent or reverse the inhibition of the intestinal Ca^{2+} absorption caused by a GSH depleting drug such as MEN in experimental animals. We have also tested the possible involvement of the antioxidant and antiapoptotic properties of the aminoacid in the gut response.

2. Material and methods

2.1. Chemicals

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.2. Animals

One-day-old Cobb Harding chicks (*Gallus gallus domesticus*) were purchased from Avícola Bartolucci Alberto and Daniel S.H, Cno San Carlos Km 5 1/2 Córdoba, Argentina, and were maintained under constant temperature (24 ± 2 °C), synchronized to a 12:12 light/dark cycle (light from 7:00 a.m. to 7:00 p.m.). They were fed a commercial normal avian diet (GEPSA Feeds, Pilar, Córdoba, Argentina) and water "*ad libitum*". At 4 weeks of age, chicks were divided into different groups: 1) controls, which received by gavage 1 mL of 0.15 M NaCl or saline solution (SS), 30 min before sacrifice, 2) treated with MEN. Chicks received an i. p. injection of 2.5 μmol of MEN/kg of b.w. 30 min before sacrifice, 3) treated with GLN. Chicks received a unique dose by gavage of GLN (the dose was dependent on the experiment), 30 min before sacrifice, and 4) treated with GLN + MEN. Chicks were sequentially treated with GLN (the dose was dependent on the experiment) by gavage and MEN (i.p. injection of 2.5 μmol of MEN/kg of b.w. 30 min before sacrifice). In some experiments, the time of exposure to the treatment was different (see data). All animals were killed by cervical dislocation and the excised duodena were rinsed with cold 0.15 M NaCl and mucosa was scraped to make homogenates or the enterocytes were isolated, as described below. The chosen dose of MEN was diluted in 0.15 M NaCl and corresponds to that dose capable of inhibiting intestinal Ca^{2+} absorption after 30 min injection, as previously described (Marchionatti et al., 2003). In a different set of experiments, some animals received MEN (2.5 μmol of MEN/kg of b.w.) plus GLN (0.5 g/kg b.w.) simultaneously or MEN (2.5 μmol of MEN/kg of b.w. for 30 min) followed by GLN (0.5-1 g/kg b.w.) for 30 min. In all cases, chicks were fasted for 24 h and the sacrifice was made between 10:30 a.m. and 12:30 a.m. The protocol was approved by the CICUAL (Res. 16/15, Commission for Care and Use of Laboratory Animals, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba). All efforts were made to minimize the number of animals used and their suffering.

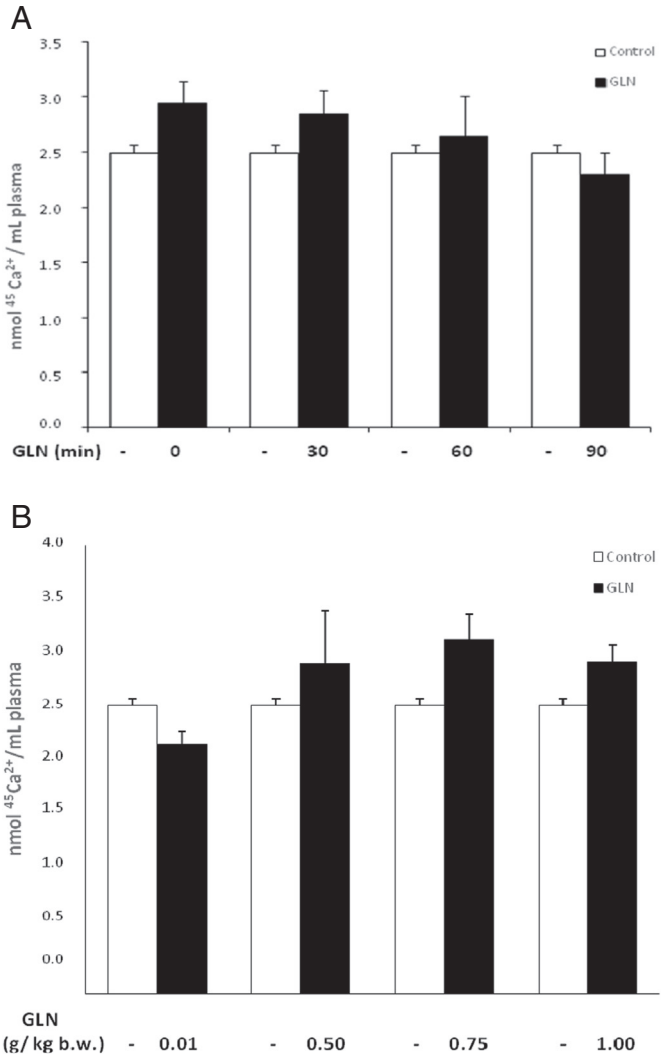


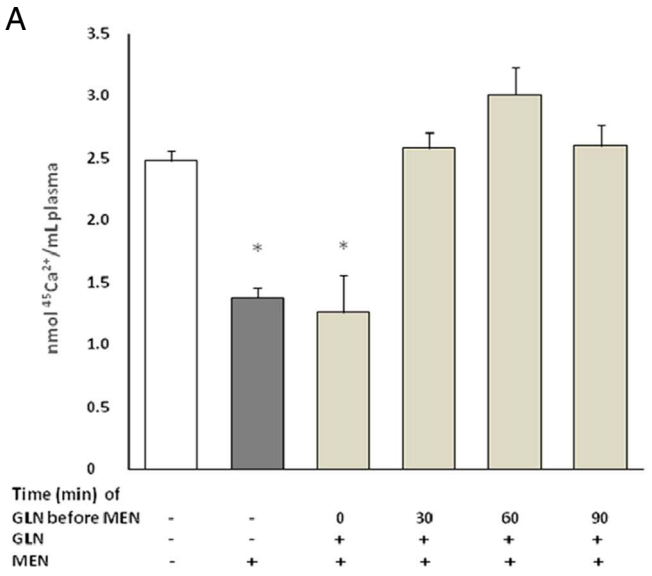
Fig. 1. Time and dose effects of GLN alone on the chick intestinal Ca^{2+} absorption. A) 0.5 g GLN/kg of b.w. at different times prior to the sacrifice, B) GLN at different doses administered 30 min before sacrifice. Values are means ± S.E. of samples from 4 to 6 animals for each experimental condition. Statistical analysis was determined by ANOVA followed by the Tukey *post hoc* test.

2.3. Intestinal Ca^{2+} absorption

We used the intestinal loop technique ligated *in situ*, as previously described (Tolosa de Talamoni et al., 1996) employing $^{45}Ca^{2+}$ as a tracer. To do this, chicks were laparotomized under anesthesia (50 mg/kg b.w. ketamine and 10 mg/kg b.w. xylazine) and a 10 cm segment of duodenum was ligated. One milliliter of 150 mM NaCl, 1 mM $CaCl_2$, plus 1.85×10^5 Bq $^{45}Ca^{2+}$, pH 7.2, was introduced into the lumen of the ligated intestinal segment. After 30 min, blood was withdrawn by cardiac puncture, centrifuged and the plasma $^{45}Ca^{2+}$ was measured in a liquid scintillation counter. Absorption was defined as an appearance of $^{45}Ca^{2+}$ in plasma.

2.4. Duodenal mature enterocytes isolation

Epithelial cells from the duodenum were isolated, as previously described (Centeno et al., 2004). Cells were collected by centrifugation at 500 × *g* for 5 min, and were resuspended in an incubation medium containing 140 mM NaCl, 5 mM KCl, 20 mM HEPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM glucose, pH 7.4. Cell maturation was determined by assaying the alkaline phosphatase (EC 3.1.3.1.) activity, as previously reported (Walter and Schütt, 1974). In various experiments,



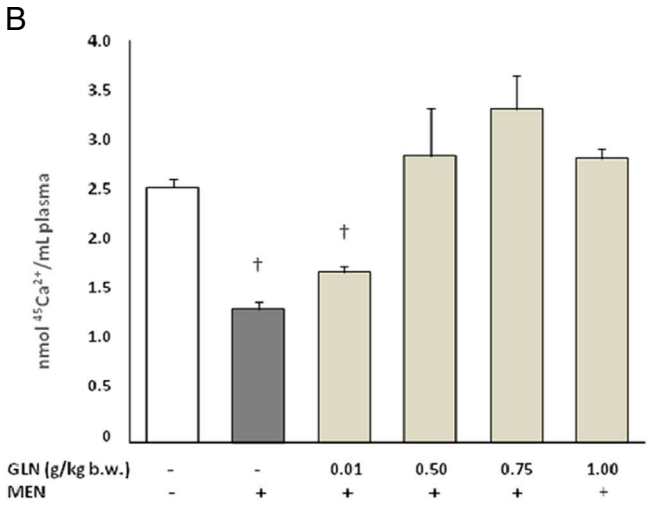


Fig. 2. Time and dose effects of GLN on the chick intestinal Ca^{2+} absorption in the absence or presence of MEN. Values are means ± S.E. of samples from 4 to 6 animals for each experimental condition. Statistical analysis was determined by ANOVA followed by the Tukey *post hoc* test. A) Time effects: 0.5 g GLN/kg of b.w. at different times before i.p. injection of 2.5 μmol of MEN/kg of b.w., *$p < 0.05$ *vs* Control and GLN (30, 60 and 90 min) + MEN. B) Dose effects: GLN at different doses administered 30 min before i.p. injection of 2.5 μmol of MEN/kg of b.w. †$p < 0.05$ *vs* Control and GLN (0.50, 0.75 and 1.00 g/kg of b.w.) + MEN.

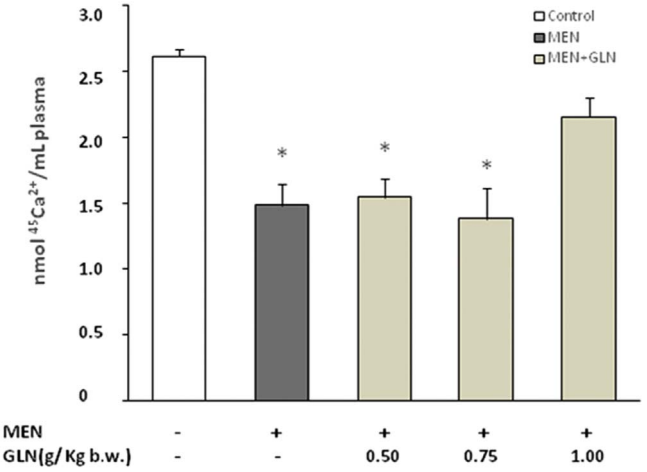


Fig. 3. Effect of GLN on the chick intestinal Ca^{2+} absorption after MEN administration. Variable doses of GLN administered at different times after i.p. injection of 2.5 μmol of MEN/kg of b.w. Values are means ± S.E. of samples from 4 to 6 animals for each experimental condition. Statistical analysis was determined by ANOVA followed by the Tukey *post hoc* test. *$p < 0.05$ *vs* Control and MEN + GLN (1 g/kg of b.w.).

only mature cells (tip cells) were used, since they are mainly involved in the intestinal Ca^{2+} absorption. Cell viability was determined by the Trypan blue exclusion technique.

2.5. Spectrophotometric procedures

Superoxide dismutase (Mg^{2+}-SOD; EC 1.15.1.1) and catalase (CAT; EC 1.11.1.6) activities were performed in diluted aliquots of the supernatants from duodenal homogenates (1:5). Mg^{2+}-SOD activity was determined in 1 μM EDTA, 50 mM potassium phosphate buffer, pH 7.8, 13 mM methionine, 75 μM nitro blue tetrazolium (NBT) and 40 μM riboflavine. SOD activity was defined in terms of its ability of inhibiting the superoxide anion ($\cdot O_2^-$)-dependent reaction due to the competition between SOD and NBT (Beauchamp and Fridovich, 1973). CAT activity was assayed in 50 mM potassium phosphate buffer pH 7.4 and 0.3 M H_2O_2. The H_2O_2 decomposition rate was directly proportional to enzyme activity (Aebi, 1974). Total GSH content was also assayed in supernatants from intestinal homogenates. The determinations were carried out by the glutathione disulfide reductase-5,5′-dithiobis (2-nitrobenzoate) recycling procedure (Anderson, 1985). The protein carbonyl content was determined by using 2,4-dinitrophenylhydrazine in an aliquot from homogenates of scraped duodenal mucosa diluted in an isolation buffer (50.3 mM HEPES, 127 mM KCl, 1.36 mM EDTA, 0.5 mM MgSO4, 0.183 mM PMSF, pH 7.4), according to the procedure of Levine et al. (1990). The $\cdot O_2^-$ measurements were accomplished in mature enterocytes. Cells were washed twice with Hanks buffer (137 mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 6.24 mM glucose, pH 7.4) and incubated with NBT (1 mg/mL) at 37 °C for 1 h. The formazan precipitates formed were dissolved in dimethylsulfoxide and quantified at 560 nm. OD values are direct indicators of $\cdot O_2^-$ concentration in the samples (Serrander et al., 2007). Caspase 3 (EC 3.4.22.56) activity was measured in supernatants from homogenates of enterocytes following the absorbance of *p*-nitroaniline obtained from a caspase 3-substrate I (Calbiochem, San Diego, CA, USA) in a plate reader at 405 nm (Garcia-Calvo et al., 1998).

2.6. Western blot analysis

Immunoblotting analysis of plasma membrane Ca^{2+}-ATPase ($PMCA_{1b}$), calbindin D_{28k} (CB D_{28k}), FAS and FASL was performed using mucosal homogenates from chick duodenum. Suspensions were done in RIPA (radio immuno precipitation assay buffer) lysis buffer (1% SDS, 0.5% sodium deoxycholate in PBS, containing 1 mM PMSF and 1 M NaF), and then centrifuged. Later, proteins (100 μg) were denatured for 5 min at 95 °C and separated in 12% (w/v) SDS-polyacrylamide minigels for CB D_{28k}, FAS and FASL and in 8% (w/v) SDS-polyacrylamide minigels for $PMCA_{1b}$ (Laemmli, 1970). Gels containing the separated proteins were immersed in the transfer buffer (25 mM Tris-HCl, 192 mM glycine, 0.05% w/v SDS and 20% v/v methanol) (Towbin et al., 1979). Nitrocellulose membranes (0.45 μm) were blocked for 1.5 h with 2% w/v nonfat dry milk in 0.5 M Tris-buffered saline solution and incubated overnight at 4 °C with the specific primary antibody at 1:1000 dilution in each case. The antibodies were: anti-$PMCA_{1b}$ (human erythrocyte clone 5F10 A7952 SIGMA Saint Louis, Missouri, USA), anti-CB D_{28k} (polyclonal antibody produced in rabbit, SIGMA Saint Louis, Missouri, USA), mouse anti-CD95 monoclonal antibody (BD Pharmigen Biosciences, San José, CA, USA), and rabbit anti-FASL polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After three washings, appropriate secondary

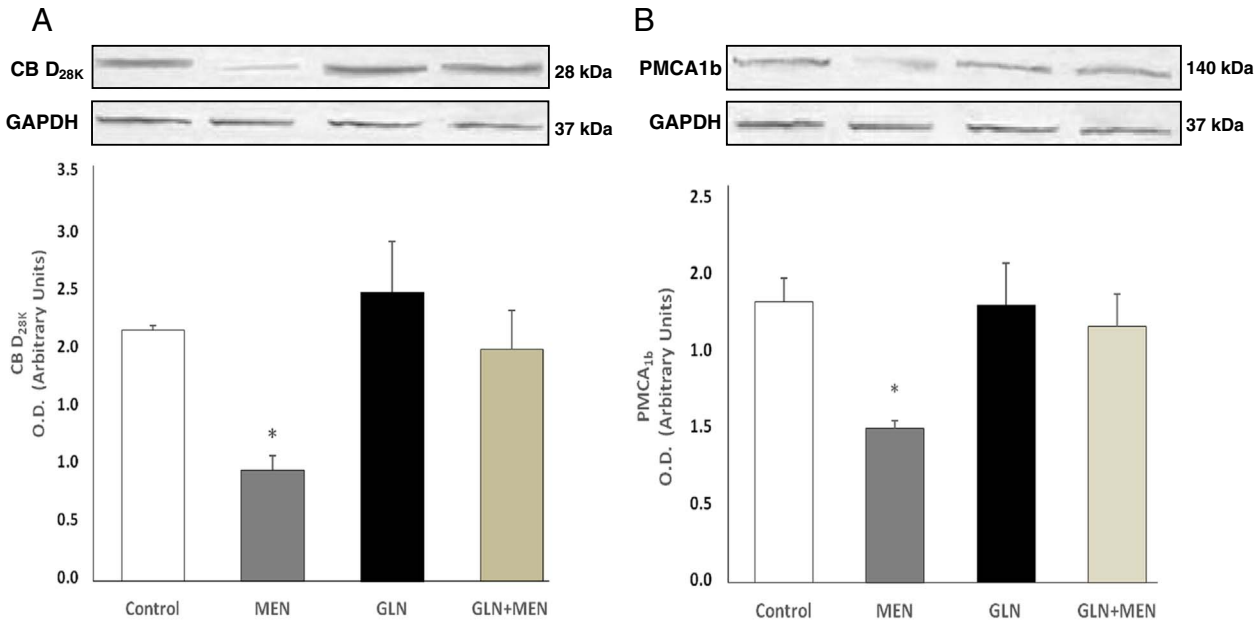


Fig. 4. Effect of GLN alone or GLN *plus* MEN on the protein expression of CB D_{28k} (A) and $PMCA_{1b}$ (B) in chick intestine. Chicks were treated with 2.5 μmol of MEN/kg b.w. or with 0.5 g GLN/kg of b.w. or with GLN 30 min prior MEN. After 30 min of treatment, the protein expression of calbindin D_{28k} (CB D_{28k}) and plasma membrane Ca-ATPase ($PMCA_{1b}$) was analyzed by Western blot in mucosa from chick duodena. Three independent experiments were accomplished. Values are means ± S.E. of samples from 4 to 6 animals for each experimental condition. Statistical analysis was determined by ANOVA followed by the Tukey *post hoc* test. *p < 0.05 *vs* Control, GLN and GLN + MEN.

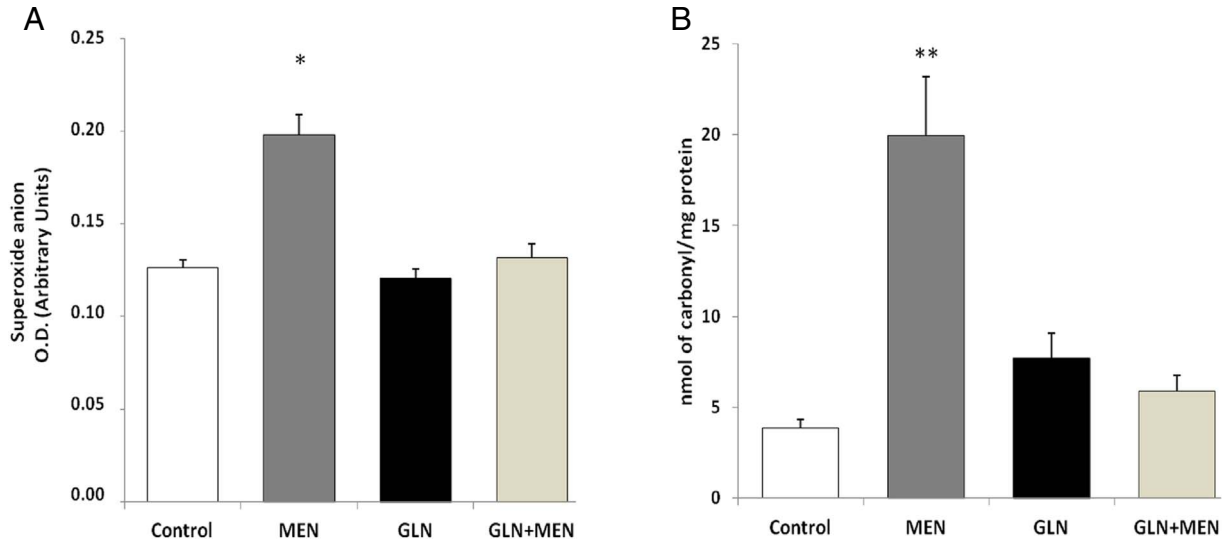


Fig. 5. Effect of GLN alone or GLN *plus* MEN on superoxide anion (A) and carbonyl content (B) from chick intestine. Chicks were treated with 2.5 μmol of MEN/kg b.w. or with 0.5 g GLN/kg of b.w. or with GLN 30 min prior MEN. The determinations were assayed in supernatants from duodenal mucosa homogenates. Values are means ± S.E. of samples from 4 to 6 animals for each experimental condition. Statistical analysis was determined by ANOVA followed by the Tukey *post hoc* test. *p < 0.001 *vs* Control, GLN and GLN + MEN. **p < 0.05 *vs* Control, GLN and GLN + MEN.

biotinylated antibodies were incubated at room temperature for 1 h. Then, the blots were washed three times and streptavidin-biotin conjugate (Histostain-SP Broad Spectrum, Invitrogen CA, USA) was added. Detection was performed using 3,3′-diaminobenzidine (DAB) as a chromogen. Monoclonal antibody anti-GAPDH (clone GAPDH-71.1) from Sigma-Aldrich, St. Louis, MO, USA, was used to detect GAPDH as a marker to normalize the relative expression of other proteins. The band intensities were quantified using an Image Capturer EC3 Imaging System, Launch VisionWorks LS software (Life Science, Cambridge, UK) in order to obtain the relative expression of proteins.

2.7. TUNEL assay

Chick duodena were fixed in 4% paraformaldehyde in 0.01 M sodium phosphate buffer pH 7.3 and sections of 5 μm were obtained. Tissue morphology was visualized and analyzed after hematoxylin-eosin staining. DNA fragmentation was measured by the terminal transferase-mediated dUTP nick-end labeling procedure (TUNEL) employing ApopTag Plus peroxidase *in situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA). The apoptotic index, expressed as the percentage of TUNEL (+) cells in relation to the total number of cells, was calculated in order to determine the magnitude of

Table 1
Effect of GLN and MEN on the glutathione content and the enzyme activities of the antioxidant system from chick intestine.

Group	GSH	Catalase	SOD
	nmol/mg protein	IU/mg protein	IU/mg protein
	(5)	(7)	(5)
Control	8.72 ± 0.06	14.62 ± 0.23	10.26 ± 0.44
MEN	4.00 ± 0.31*	23.41 ± 1.19**	21.97 ± 1.95**
GLN	8.42 ± 1.58	12.33 ± 1.35	11.22 ± 1.19
GLN + MEN	7.98 ± 0.26	11.29 ± 1.35	10.37 ± 0.51

The animals were treated by gavage with 0.5 g GLN/kg of b.w. and 30 min later they were injected i.p. with 2.5 μmol MEN/kg of b.w. After half an hour they were sacrificed. Data are means ± S.E. ( ) = number of animals in each group. Statistical analysis was determined by ANOVA followed by the Tukey *post hoc* test.

* $p < 0.05$ *vs* control, GLN and GLN + MEN.

** $p < 0.001$ *vs* control, GLN and GLN + MEN.

the apoptotic process. The apoptotic index was assessed by counting ten villi at 400× magnification from 3 chicks for each treatment. The counting was accomplished by three independent researchers in a blinded fashion.

2.8. Statistics

Data are expressed as means ± SE. Results were evaluated by one-way analysis of variance (ANOVA) and the Tukey's test as a *post hoc* test. Differences were considered statistically significant at $p < 0.05$. All the analyses were carried out by using SPSS software (version 22.0) for Windows 8.1 (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Time and dose effects of GLN on chick intestinal Ca^{2+} absorption in the absence or presence of MEN

The treatment of GLN alone did not affect the intestinal Ca^{2+} absorption in one-month old chicks. This lack of effect was observed at different times of administration as well as after using variable doses of GLN (Fig. 1A and B). However, GLN administration prior to MEN treatment blocked the inhibition of the intestinal Ca^{2+} absorption caused by the quinone, leading the cation absorption to the control values. The protective effect of GLN was observed when the aminoacid was administered at least 30 min before the MEN injection. When GLN and MEN were given simultaneously, the blocking effect of GLN did not occur (Fig. 2A). A dose of 0.01 g GLN/kg of b.w. 30 min before MEN injection was not enough to block the inhibitory effect of the quinone; doses of 0.5 g/kg of b.w. or higher prevented the inhibitory effect of MEN (Fig. 2B). When GLN was administered after MEN injection, the aminoacid returned the intestinal Ca^{2+} absorption to control values only at doses of 1 g/kg of b.w. (Fig. 3).

3.2. GLN blocked the inhibitory effect caused by MEN on protein expression of molecules involved in the transcellular pathway of chick intestinal Ca^{2+} absorption

As previously shown, the protein expression of two molecules involved in the transcellular pathway of intestinal Ca^{2+} absorption, such as CB D_{28k} and the $PMCA_{1b}$ from chick duodenal mucosa, was inhibited by MEN (Areco et al., 2016). GLN by itself did not alter the protein expression of both molecules; however, the previous administration of 0.5 g GLN/kg of b.w. to MEN injection blocked the inhibitory effect of the quinone (Fig. 4A and B).

3.3. GLN avoided changes in the chick intestinal redox state provoked by MEN

In agreement with previous data, MEN enhanced the superoxide anion content and the protein carbonyl groups of chick duodenal mucosa (Areco et al., 2016). Both effects were avoided by the previous GLN treatment, but GLN alone produced similar values to the control ones (Fig. 5A and B). With regard to the GSH content from chick intestinal mucosa, MEN produced depletion, which was avoided by the GLN treatment prior to the quinone injection. However, GLN alone did not modify the GSH content in the chick intestine (Table 1). The activities of SOD and CAT, two enzymes of the antioxidant system, were increased by MEN, effects that were blocked by pretreatment of GLN. When GLN was administered alone, the enzyme activities were similar to those from the control group (Table 1).

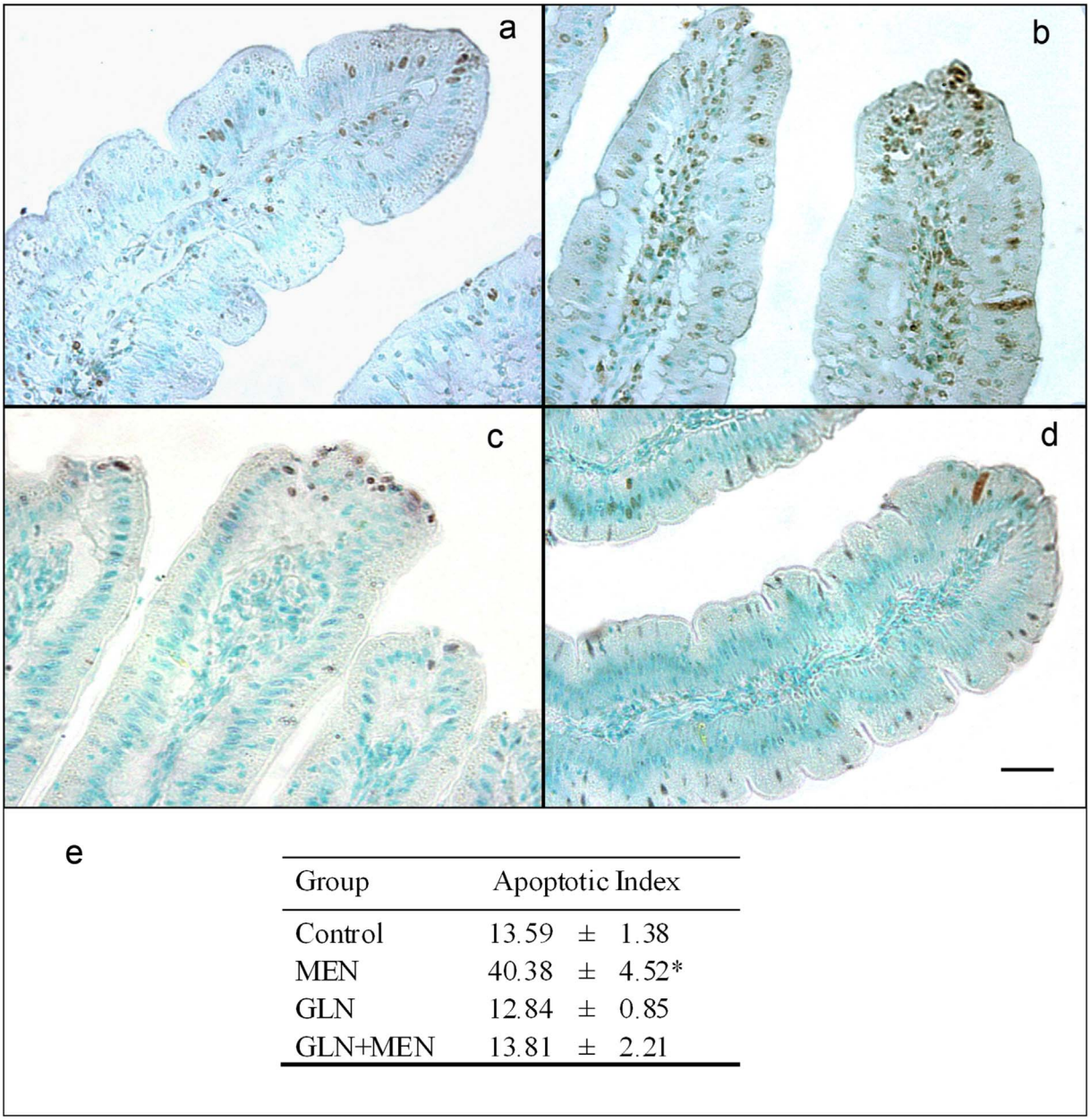
3.4. GLN abrogated apoptotic effects caused by MEN in the chick intestinal mucosa

As depicted in Fig. 6, MEN increased cell death by apoptosis in chick intestinal mucosa, as judged by the enhancement in the number of TUNEL (+) cells. GLN did not alter the DNA fragmentation as compared to the control group, but GLN blocked the increment in the number of TUNEL (+) cells caused by MEN, which can be more appreciated with the values of the apoptotic index (Fig. 6). An increase in the protein expression of FAS and FASL and in the caspase-3 activity by MEN indicates that the extracellular pathway of apoptosis was triggered by the quinone. Again, all these effects were avoided by the previous treatment of GLN, and when GLN was given alone, the data were identical to the control values (Fig. 7).

4. Discussion

The present data indicate that GLN not only prevents but also restores the inhibition of the chick intestinal Ca^{2+} absorption that occurs under oxidant conditions such as MEN exposure. Interestingly, GLN by itself does not affect the intestinal Ca^{2+} absorption, but the aminoacid protects this physiological process when the redox state in the intestine is altered. GLN protection depends on the dose and time of exposure. At least 0.5 g/kg of b.w. by gavage and 30 min in advance is necessary for GLN to show its protector effect. In case that GLN is administered after MEN injection, the dose of GLN to restore the intestinal Ca^{2+} absorption must be higher (1 g/kg of b.w.). In other words, lower doses of GLN are needed for prevention than those for restoration of the inhibitory effect of MEN on intestinal cation absorption. Noth et al. (2013) have also found that oral GLN supplementation for 6 days restores intestinal permeability dysfunction in a murine acute graft-*vs*-host disease model. The beneficial effects of GLN in a post ischemic gut were lost in syndecan-1 knockout mice. The authors of this study suggest that GLN restores the intestinal permeability, at least partially, through an enhancement of cell surface levels of syndecan-1, a predominant heparan sulfate proteoglycan found on the surface of epithelial cells, which is essential for maintaining the intestinal epithelial barrier (Peng et al., 2012).

The protector effect of GLN on the intestinal Ca^{2+} absorption is accomplished through restoration of protein expression of molecules involved in the transcellular Ca^{2+} pathway, as suggested by normalization of the protein expression of CB D_{28k} and $PMCA_{1b}$. Therefore, one possibility would be that GLN stimulates the translation of these proteins involved in the transcellular Ca^{2+} pathway. Using microarray analysis, Wang et al. (2008) have observed that GLN increases the expression of genes that are crucial for intestinal growth and antioxidant capacity in weanling pigs; although how these transcriptional alterations translate into enhanced gut growth and function remains unknown. *In vitro* and *in vivo* studies have demonstrated that GLN acts as a protector by enhancing heat shock protein (HSP) expression (Morrison



Group	Apoptotic Index
Control	13.59 ± 1.38
MEN	40.38 ± 4.52*
GLN	12.84 ± 0.85
GLN+MEN	13.81 ± 2.21

Fig. 6. Effect of GLN alone or GLN *plus* MEN on the apoptosis of villus tip cells from chick duodenum. Chicks were treated with 2.5 μmol of MEN/kg b.w. or with 0.5 g GLN/kg of b.w. or with GLN 30 min prior MEN. DNA fragmentation was determined by the TUNEL assay. a) Control, b) MEN treated, c) GLN treated, and d) GLN + MEN treated. Bar: 20 μm. e) Apoptotic index in villus tip cells, which was assessed by counting ten villi at 400 × magnification from 3 chicks for each treatment. Values are means ± S.E. of samples from 4 to 6 animals for each experimental condition. Statistical analysis was determined by ANOVA followed by the Tukey *post hoc* test.*$p < 0.001$ *vs* Control, GLN and GLN + MEN.

et al., 2006; Singleton and Wischmeyer, 2007); however, the pathway by which GLN induces HSP seems to be complex and multifaceted (Niederlechner et al., 2013a). Hamiel et al. (2009) have shown that GLN enhances hexosamine biosynthetic pathway leading to the *O*-glycosylation, nuclear translocation, and transcriptional activation of heat shock factor-1 and Sp1, both of which are key transcription factors required for maximal HSP70 expression. In IEC-6 cells, it has been shown that GLN activates ERK1/2 and dephosphorylates p38MAPK after heat stress (Niederlechner et al., 2013b). Since the activation of ERK1/2 by GLN is rapid, 10 to 20 min (Rhoads, 1999), it is quite possible that GLN improves the intestinal Ca^{2+} absorption through stimulation of ERK1/2. However, Wu et al. (2015) have shown that advanced oxidation products decrease the expression of Ca^{2+} transport molecules in small intestinal epithelium *via* extracellular signal-regulated kinases p44/42 MAPK signaling pathway.

GLN blocks the oxidative stress caused by MEN, as indicated by normalization of the protein carbonyl groups as well as the GSH and $\cdot O_2^-$ contents in the chick duodenum. We have previously demonstrated that GSH depleting drugs cause inhibition of the intestinal Ca^{2+} absorption (Marchionatti et al., 2003; Tolosa de Talamoni et al., 1996), effect that disappears when an antioxidant is administered (Marchionatti et al., 2013; Areco et al., 2016). In fact, GLN preserved the intestinal GSH content when animals were exposed to MEN, as did in conditions of intestinal I/R (Harward et al., 1994). However, GLN alone does not modify the intestinal GSH content. In lung cancer cell lines, it has been demonstrated that a significant amount of extracellular GSH is directly derived from GLN, but the 50% of the GSH synthesized that comes from GLN-derived glutamate is produced after 12 h (Sappington et al., 2016). Since our study has been accomplished at shorter times, it is quite possible that the GSH synthesis was not high enough to be detected; maybe this could occur at longer times. The activities of SOD and CAT are increased by MEN, probably as a

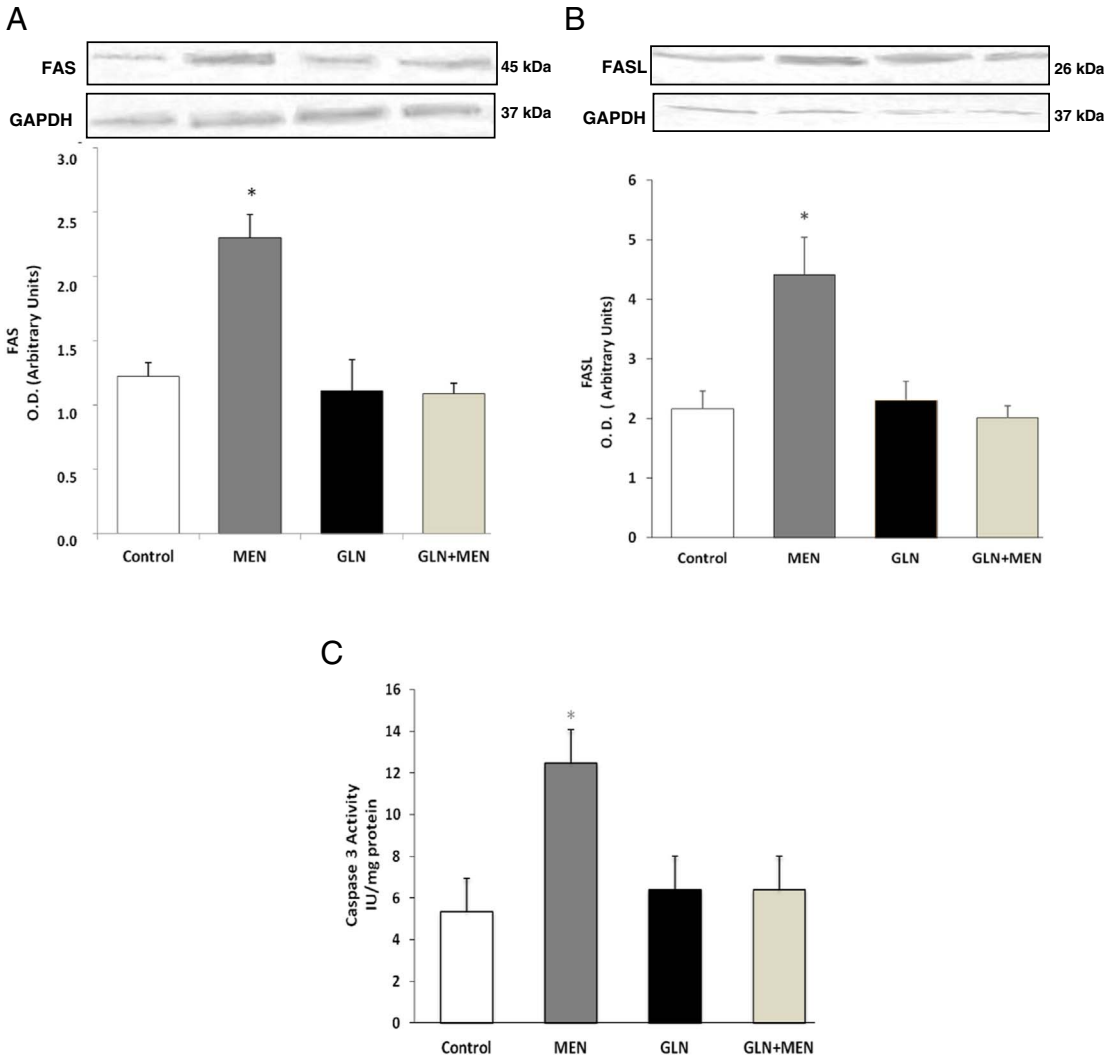


Fig. 7. Effect of GLN alone or GLN *plus* MEN on the protein expression of FAS (A) and FASL (B), and the caspase 3 activity (C) in chick intestine. Chicks were treated with 2.5 μmol of MEN/kg b.w. or with 0.5 g GLN/kg of b.w. or with GLN 30 min prior MEN. Values are means ± S.E. of samples from 4 to 6 animals for each experimental condition. Statistical analysis was determined by ANOVA followed by the Tukey *post hoc* test. *p < 0.05 *vs* Control, GLN and GLN + MEN.

compensatory effect on the oxidative stress. Although GLN alone is unable to modify the activity of both enzymes, in the presence of MEN either SOD or CAT activity are restored to the control values by GLN. The normalization of SOD and CAT activities by GLN under oxidant conditions has also been observed in the intestine of juvenile Jian carp (Jiang et al., 2015).

Another mechanism by which GLN avoids the inhibition of intestinal Ca^{2+} absorption might be by blocking MEN-induced apoptosis. It is well documented that in rat intestinal epithelial cells, the GLN-deprivation causes significant induction of apoptosis with increased Annexin V staining, DNA laddering, and nuclear condensation (Papaconstantinou et al., 1998). GLN has been shown to decrease the hyperglycemia + hypoxia/reoxygenation induced cardiomyocyte apoptosis and decrease I/R injury in diabetic rats partly through down-regulation of TGF-β1-Smad3 pathway activation (Zhang et al., 2016). In fish erythrocytes, GLN has prevented hydroxyl radical-induced apoptosis through inhibition of mitochondria and calcium ion involved pathways (Li et al., 2016). In our case, GLN blocks the enhancement in protein expression of FAS, FASL and caspase-3 activity caused by MEN, which means that GLN prevents the triggering of the extrinsic pathway of apoptosis provoked by the quinone. FAS is expressed on the surface of a number of cell types, and when FAS interacts with its natural ligand FASL, apoptosis is induced. GLN avoids the enhancement of the protein expression of both molecules produced by MEN through an unknown mechanism. In addition, GLN blocks the increment in the caspase-3 activity, which is necessary for apoptotic chromatin condensation and DNA fragmentation in all cell types (Exner et al., 2002). In mice treated with zymosan, the development of intestinal injury was also attenuated by GLN, at least in part, as a consequence of its reduction in the FASL expression and in the number of TUNEL (+) cells (Mondello et al., 2011). Similarly, in our study the increment in the number of TUNEL (+) cells caused by MEN was blocked by GLN previous administration. The antiapoptotic effects of GLN in intestinal HT-29 cells exposed to TRAIL-induced apoptosis were independent of DNA/RNA synthesis. Apparently, GLN prevented the cytokine-induced apoptosis *via* the pirimidine pathway (Evans et al., 2005). As shown, GLN has anti-apoptotic properties, which occur through several mechanisms. The antiapoptotic role of GLN against the MEN effect on the intestinal Ca^{2+} absorption involves abolition of the activation of the system FAS/FASL/caspase-3. It is quite possible that other antiapoptotic pathways are also triggered by GLN.

To conclude, the data indicate that under oxidant conditions GLN protects the intestinal Ca^{2+} absorption by maintaining the expression of the transporters involved in the intestinal transcellular pathway. The protective effect may be achieved because GLN restores the intestinal GSH levels, normalizes the enzymatic activities of the antioxidant

system and reduces the activation of the apoptotic pathway FAS/FASL/caspase-3. Therefore, GLN could be an oral nutritional supplement of choice to normalize the redox state and the proliferation/cell death ratio in the small intestine improving the intestinal Ca^{2+} absorption and, probably other intestinal functions altered by oxidative stress.

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