

# Acute and chronic acidosis influence on antioxidant equipment and transport proteins of rat jejunal enterocyte

Marisa Tosco<sup>1\*</sup>, Cristina Porta<sup>†</sup>, Chiara Sironi<sup>†</sup>, Umberto Laforenza<sup>‡</sup> and Maria Novella Orsenigo<sup>\*</sup>

<sup>\*</sup> Dipartimento di Scienze Biomolecolari e Biotecnologie, Università di Milano, I-20133 Milano, Italy

<sup>†</sup> Dipartimento di Fisiologia Umana, Università di Milano, I-20133 Milano, Italy

<sup>‡</sup> Dipartimento di Fisiologia, Università di Pavia, I-27100 Pavia, Italy

## Abstract

Acidosis elicits the formation of oxidants and, in turn, ROS (reactive oxygen species)-induced intestinal diseases cause acidosis. This research investigated whether both acute and chronic acidosis influence the antioxidant enzymatic equipment of rat jejuncocyte, including  $\gamma$ -GT activity, involved in GSH (glutathione) homeostasis. Lipid peroxidation level and the expressions of (Na<sup>+</sup>, K<sup>+</sup>)-ATPase and GLUT2 were also investigated. The possible influence of acidosis on ROS action was tested. Isolated apical membranes, everted sac preparations and homogenates from acidotic rats were used.  $\gamma$ -GT activity is inhibited after incubation of isolated membranes at acidic pH, but using the whole intestinal tract this inhibition disappears, while SOD (superoxide dismutase) and GR (glutathione reductase) activities are enhanced. Also, in conditions of chronic acidosis,  $\gamma$ -GT activity is unaffected, but no variations of antioxidant activities are apparent. (Na<sup>+</sup>, K<sup>+</sup>)-ATPase expression increases, while GLUT2 decreases in acidotic animals. Lipid peroxidation level is unaffected by acidosis. H<sub>2</sub>O<sub>2</sub> inhibits  $\gamma$ -GT activity only in isolated membranes; in the whole tissue, it enhances CAT (catalase) and SOD activities and reduces GLUT2 expression. The pattern of responses to oxidant agents is unaffected by acidosis. Although jejunum seems quite resistant to acidosis, results, suggesting specific responses to this condition, may direct further research on antioxidant supplementation.

Keywords:  $\gamma$ -GT; acidosis; antioxidant enzyme; GLUT2; (Na<sup>+</sup>/K<sup>+</sup>)-ATPase; rat jejunum

## 1. Introduction

Acidosis promotes lipid peroxidation or other manifestations of oxidant-mediated damage in various cell types (Aranow and Fink, 1996; Cutaia et al., 2002; Hassan et al., 2009; Alva et al., 2010); moreover, a number of studies have implicated the occurrence of acidosis in ROS (reactive oxygen species)-induced intestinal inflammatory diseases (Halliwell and Gutteridge, 1999; Prabhu et al., 2000; Rahman et al., 2006). Acidosis associated with the inflammatory condition, in turn, produces oxidative stress and/or amplifies its effects (Pedoto et al., 2001; Antonova et al., 2009; Hassan et al., 2009; Hazell, 2009); *in vivo* and *in vitro* studies indicate that at an acidotic pH, the response of the gut to an insult is magnified (Homma et al., 2005).

It has been reported that in the proximal tubule a mechanism whereby acidosis enhances oxidant-mediated damage is inhibition of two enzymes involved in GSH (glutathione) homeostasis, GR (glutathione reductase) and GPx (glutathione peroxidase) (Aranow and Fink, 1996). Another key enzymatic activity for the maintenance of GSH homeostasis, and thus for the preservation of antioxidant capacity, is  $\gamma$ -GT. Breaking down extracellular GSH,  $\gamma$ -GT activity provides precursor amino acids to be assimilated and reutilized for intracellular GSH synthesis. Despite the importance of  $\gamma$ -GT on GSH homeostasis, up to now, no data are available regarding the effect of acidosis on intestinal  $\gamma$ -GT activity or on its sensitivity to various oxidant species.

In order to get more insight into the pathogenesis of the diseases in which acidotic conditions are present, we investigated whether both acute and chronic acidosis influence the antioxidant enzymatic equipment of the jejunal enterocyte, as well as some important proteins involved in cellular function.

## 2. Materials and methods

### 2.1. BBMs (brush border membranes) separation

The experiments were performed according to national ethical guidelines and approved by 'Comune di Milano – Uff. Diritti degli animali', 'Regione Lombardia' and 'Ministero della Salute' (prot. 5/2008, approval November 2008, validity 3 years). Male albino rats (Wistar strain, Charles River Italiana), weighing 250–300 g, were deeply anaesthetized with an injection of 2,2,2-tribromoethanol [0.05 g (250 g body weight)<sup>-1</sup> i.p.] and then decapitated. BBMs were isolated from rat jejuncocytes by the Mg<sup>2+</sup> precipitation method as previously described (Tosco et al., 2004). When used, 20 mM ATZ (3-amino-1,2,4 triazole) and 3 mM MS (mercaptosuccinic acid) were added before the last centrifugation. In acidosis experiments, the pellet was divided in two parts that were resuspended in 250 mM sorbitol and either 20 mM Hepes/Tris pH 7.5 or 20 mM Mes/Tris pH 6.0; BBMs were incubated in these conditions at 37°C for 60 min. Membrane fraction purity was assessed as previously described (Orsenigo et al., 1985).

<sup>1</sup> To whom correspondence should be addressed (email marisa.tosco@unimi.it).

**Abbreviations:** ATZ, 3-amino-1,2,4 triazole; BBM, brush border membrane; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide;  $\gamma$ -GT,  $\gamma$ -glutamyltransferase; MDA, malonaldehyde; MS, mercaptosuccinic acid; ROS, reactive oxygen species; SOD, superoxide dismutase; TBH, ter-butylhydroperoxide; TBST, Tris-buffered saline with Tween.

## 2.2. Oxidant exposure of BBMs

Oxidant exposure of BBMs was performed as described by Prabhu and Balasubramanian (2003). Briefly, isolated BBMs, preincubated 1:1 (v/v) at 37°C for 30 min in the resuspension solutions added with 20 mM ATZ and 3 mM MS, were incubated for further 30 min with the following substances separately: H<sub>2</sub>O<sub>2</sub> ranging from 0.1 to 3 mM, 1 and 2 mM TBH (ter-butylhydroperoxide); 1 mM GSSG (glutathione disulfide, final concentrations). As a control, corresponding membranes were incubated without the addition of oxidants. When H<sub>2</sub>O<sub>2</sub> was tested, H<sub>2</sub>O<sub>2</sub> concentration (mM) was determined according to Duvall et al. (1998) during the incubation period. All determinations were performed at least in duplicate.

In some experiments, BBMs were incubated at 37°C for 30 min in the presence of 0.1 mM xanthine+0.7 mM hypoxanthine+0.02 units/ml xanthine oxidase, with or without 0.1 mM allopurinol or 0.1 mM FeSO<sub>4</sub>. Preincubation, 30 min, was carried out to induce ROS generation in the absence of BBMs. To verify ROS formation, H<sub>2</sub>O<sub>2</sub> concentration (μM) was determined according to Klassen et al. (1994) during the whole preincubation and incubation periods (not reported). Controls containing only xanthine+hypoxanthine or xanthine oxidase were also tested. Unspecific effects of all the added substances were tested.

## 2.3. Everted sac preparation

Two segments of the upper jejunum (approximately 10 cm in length each) were removed, everted and incubated for 60 min at 28°C. This temperature was chosen since at 37°C the intestinal transport activity does not seem to be in a steady state (Faelli et al., 1979). The mucosal side of the intestine was incubated in 15 ml of Krebs–Henseleit–Tris solution at pH 7.5 (control) or 6.0 (composition in mM: 140.0 Na<sup>+</sup>, 5.9 K<sup>+</sup>, 2.5 Ca<sup>2+</sup>, 1.2 Mg<sup>2+</sup>, 127.7 Cl<sup>-</sup>, 24.9 HCO<sub>3</sub><sup>-</sup>, 12.2 SO<sub>4</sub><sup>2-</sup> and either 25 mM Hepes/Tris pH 7.5 or 25 mM Mes/Tris pH 6.0) added with 5.56 mM D-glucose. The serosal side of the intestine was incubated in 1 ml of the same solution. After 5 min preincubation, the 60-min experimental period started. Some experiments were performed in the presence of ~5 mM H<sub>2</sub>O<sub>2</sub> that was added in the mucosal solution after the first 30-min incubation. When present, H<sub>2</sub>O<sub>2</sub> concentration in the mucosal solution was measured every 5 min during the time course of the experiment and adjusted to 5 mM if necessary. At the end of the experiment, the intestine was blotted on filter paper and weighed.

The mucosal layer was scraped off at 0°C. A part of the scraped mucosa was used for Western blot experiments. The remaining mucosa was resuspended in 50 mM phosphate buffer, pH 7.4 (10% w/v), homogenized and tested for γ-GT and (Na<sup>+</sup>, K<sup>+</sup>)-ATPase activities. The protein content was determined by the method of Bradford (1976). The remaining homogenate was centrifuged at 10000 g for 10 min at 4°C in a refrigerated centrifuge (Hermle). The supernatant so obtained was used for the assays of SOD, CAT, GPx, GR. For the estimation of lipid peroxidation, homogenate was prepared in chilled 1.15% (w/v) KCl solution.

## 2.4. Markers of oxidative stress

### 2.4.1. Antioxidant enzymes

CAT activity was measured according to the method of Aebi (1983) by following the decrease in absorbance of H<sub>2</sub>O<sub>2</sub> at 240 nm for 5 min.

SOD activity was measured by the inhibition of pyrogallol autooxidation at 420 nm according to Guzik et al. (2005).

GPx activity was measured by following the oxidation of NADPH at 340 nm according to Anwer et al. (2007).

GR activity was measured as a decrease in absorbance of NADPH for 5 min at 340 nm according to Ojano-Dirain et al. (2005).

### 2.4.2. Lipid peroxidation

MDA production was assessed spectrophotometrically with the method defined by Ohkawa et al. (1979).

## 2.5. Induction of metabolic acidosis in rats

Rats were maintained on standard chow and had access to drinking water *ad libitum*. To induce metabolic acidosis, rats were given 0.28 M NH<sub>4</sub>Cl in the drinking water for 7 days. Body weights of the animals were recorded on the first and last days. After the treatment, the animals were killed under anaesthesia, always between 09:00 and 10:00 a.m. to avoid any possible cyclic daily variations in antioxidant levels. To confirm acidosis, blood pH was measured immediately before death directly from blood in the left ventricle. The intestinal tissues were dissected and the scraped mucosa was weighed, rapidly freeze-clamped at liquid nitrogen temperature and stored at -80°C until use.

## 2.6. Protein extraction and Western blot

After the everted sac experiments described above, the jejunal mucosa was scraped off, and total proteins were extracted. Briefly, the scraped material from each rat was resuspended in cold buffer sucrose–histidine (IS) containing 0.3 M sucrose, 25 mM histidine, 1 mM EDTA, supplemented with protease inhibitors (Roche), homogenized and then centrifuged at 4°C for 15 min at 5000 g. The supernatant was recovered, protein concentration was measured (Bradford 1976) and equal amounts of proteins (5 μg for Na<sup>+</sup>, K<sup>+</sup>-ATPase and 60 μg for GLUT2) were analysed by 7% SDS/PAGE electrophoresis. The proteins transferred to a PVDF membrane (Miniprotean 3, Biorad), were probed overnight at 4°C with the specific primary antibodies. In particular, a monoclonal anti-ATPase alpha 1 (Na<sup>+</sup>/K<sup>+</sup>) (Novus Biologicals) diluted 1:5000 in 5% non-fat dry milk–TBST (Tris-buffered saline with Tween) buffer and the polyclonal anti-GLUT2 (Chemicon) diluted 1:1000 in 5% non-fat dry milk–TBST buffer were used. The primary antibody for GLUT2 was detected with a goat anti-rabbit IgG conjugated to horseradish peroxidase (Chemicon) used at a 1:40000 dilution in 5% non-fat dry milk TBST buffer, while the anti-ATPase alpha 1 (Na<sup>+</sup>,K<sup>+</sup>) was detected with a goat anti-mouse IgG conjugated to horseradish peroxidase (Santa Cruz Biotech) diluted 1:3000 in 5% non-fat dry milk TBST buffer. Sites of antibody–antigen reaction were visualized by using Amersham ECL Plus followed by autoradiography.

## 2.7. Statistics

Statistical analysis was done by Student's *t* test or by ANOVA (analysis of variance) followed by *post hoc* Tukey's limitation. Values reported in the text are means  $\pm$  S.E.M.

## 3. Results

### 3.1. Experiments performed with isolated membranes

#### 3.1.1. Acute acidosis effects on $\gamma$ -GT activity

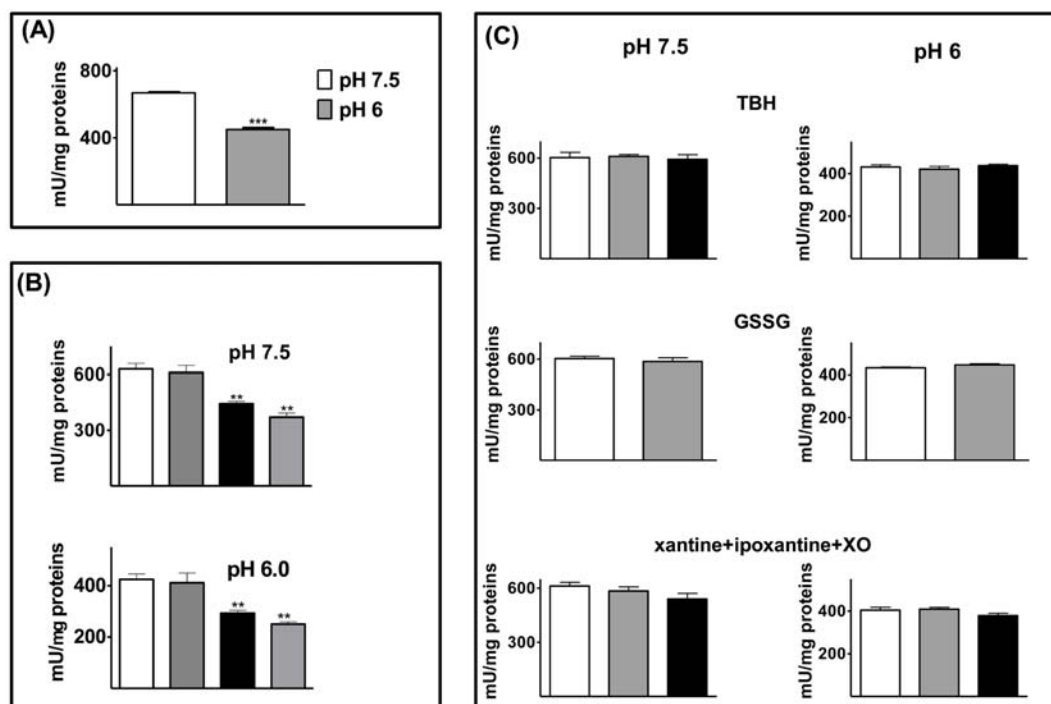
Jejunal BBMs were highly purified as reflected by about 20-fold enrichment for the BBM-specific marker enzyme  $\gamma$ -GT ( $30.56 \pm 2.28$  m-units/mg,  $n=10$ , in the homogenate;  $593 \pm 139$  m-units/mg,  $n=10$ , in the BBM fraction), while the activities of the basolateral membrane-specific marker enzyme ( $\text{Na}^+, \text{K}^+$ )-ATPase were not statistically different in the homogenate and BBM fractions. The acidic pH of the incubating solution, applied after the separation procedure, affects  $\gamma$ -GT activity; as a matter of fact,  $\gamma$ -GT activity determined in BBMs incubated at pH 6 was significantly reduced with respect to the activity measured in the same membranes resuspended at pH 7.5 (control; Figure 1A).

#### 3.1.2. $\text{H}_2\text{O}_2$ effect on $\gamma$ -GT activity at different pH values

Exposure of BBMs to different concentrations of  $\text{H}_2\text{O}_2$  (mM) resulted in a dose-dependent decrease in  $\gamma$ -GT activity; on the contrary,  $\text{H}_2\text{O}_2$  concentrations up to 200  $\mu\text{M}$  were ineffective. The results obtained at physiological pH are shown (Figure 1B), and it is evident that overlapping data were obtained at pH 6.0. Previous studies (Orsenigo et al., 2007) suggested the presence of peroxidase activities in the membrane suspensions that could be completely suppressed by 20 mM ATZ and 3 mM MS, specific inhibitors of CAT and GPx activities, respectively (Dringen and Hamprecht, 1997). Thus, these inhibitors were added during the separation procedure and in the incubating solutions.

#### 3.1.3. Effects of oxidants on $\gamma$ -GT activity at different pH values

Results indicate that the activity of  $\gamma$ -GT was not affected by TBH at both concentrations used (1 and 2 mM; Figure 1C). From data illustrated in the same figure, it is evident that no significant difference was observed in enzyme activity in the presence of 1 mM oxidized glutathione (GSSG), with respect to the control condition. Further, data illustrate the effect of  $\mu\text{M}$  concentrations of  $\text{H}_2\text{O}_2$ ,  $\text{O}^{\cdot -}$  and  $\text{HO}^{\cdot}$  on  $\gamma$ -GT activity determined by incubating BBMs, at the two different pHs, in media containing xanthine, hypoxanthine and xanthine oxidase, source of free radicals such as  $\text{H}_2\text{O}_2$  and superoxide anion (Figure 1C). The level of peroxide attained in the incubating mixture



**Figure 1**  $\gamma$ -GT response to acidosis and ROS in isolated membranes. (A) Effect of acidosis on  $\gamma$ -GT activity in isolated jejunal brush border membranes. (B) Concentration-dependent inhibitory effect of  $\text{H}_2\text{O}_2$  on  $\gamma$ -GT activity in isolated jejunal brush border membranes at physiological pH. White, control; dark grey, 130  $\mu\text{M}$   $\text{H}_2\text{O}_2$ ; black, 1.5 mM  $\text{H}_2\text{O}_2$ ; pale grey, 3 mM  $\text{H}_2\text{O}_2$ . (C) Effects of TBH, GSSG, superoxide anion and hydroxyl radicals on  $\gamma$ -GT activity in isolated jejunal brush border membranes at two different pHs. (top) white, control; dark grey, 1 mM TBH; black, 2 mM TBH; (middle) white, control; dark grey, 1 mM GSSG; (bottom) white, control; dark grey, xanthine+ipoxanthine+XO; black, xanthine+ipoxanthine+XO+ $\text{Fe}^{2+}$ . Values are means  $\pm$  S.E.M. Number of experiments=4 or 5 with duplicate estimation. \*\*\* $P < 0.0001$ , \*\* $P < 0.001$ .

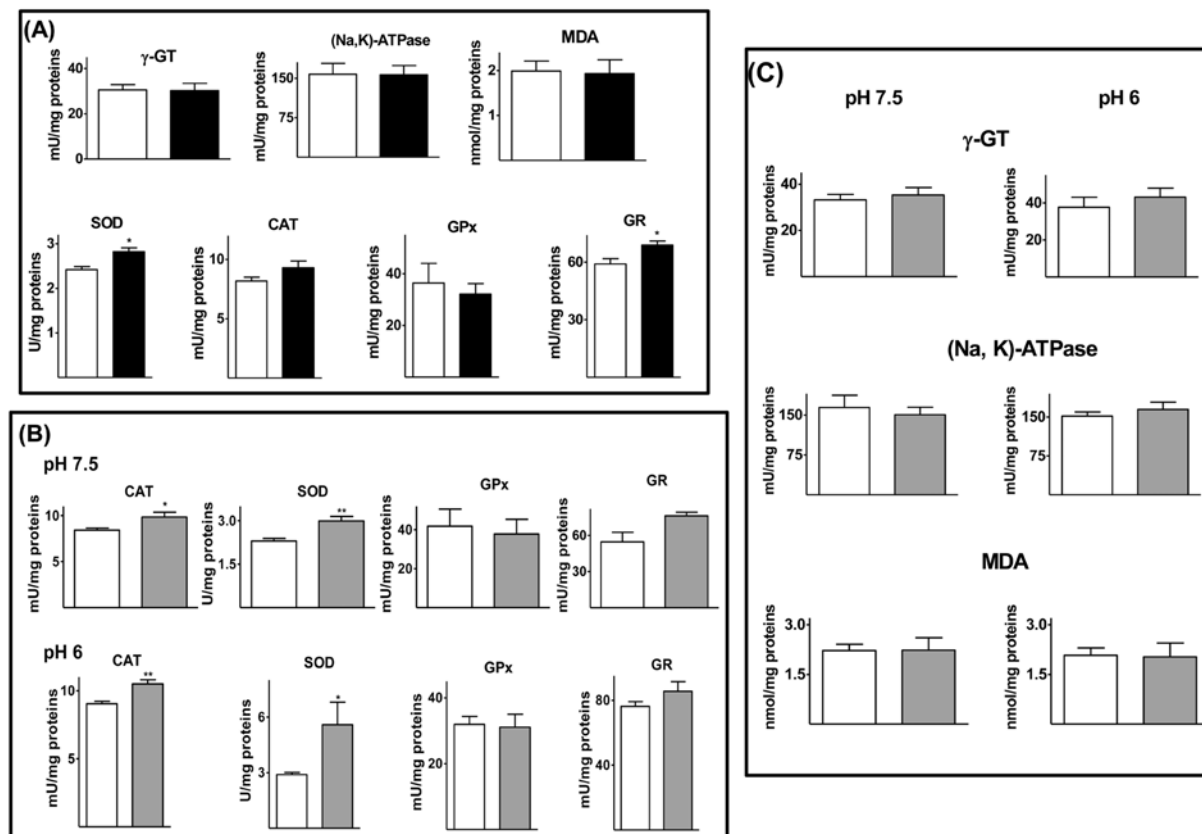
was about 200  $\mu\text{M}$  within half an hour (not reported) before the addition of BBMs (1:1 v/v). As depicted, no significant difference was evidenced for  $\gamma$ -GT activity in the presence of  $\text{H}_2\text{O}_2$  and superoxide anion with respect to the basal control, in which ROS production was prevented by inhibiting xanthine oxidase by allopurinol (Figure 1C).  $\text{H}_2\text{O}_2$  can generate hydroxyl radical in the presence of  $\text{Fe}^{2+}$  (Chatterjee et al., 2000): no changes in  $\gamma$ -GT activity were observed if  $\text{Fe}^{2+}$  was added to the incubating mixture. Overlapping results were obtained in jejunal brush border membranes incubated at the two different pHs for all the conditions examined.

## 3.2. Everted sac incubation

### 3.2.1. Acute acidosis effects on $\gamma$ -GT, $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ , antioxidant enzyme activities and MDA production of mucosal homogenate

Two contiguous pieces of the same intestinal tract, everted and incubated *in vitro*, were exposed for 60 min to incubating solutions buffered either at pH 7.5 (control) or at pH 6.0 (acidic

condition). Our results (Figure 2A) demonstrate that an acute direct exposure of intestine to acidic pH does not influence the activities of apical  $\gamma$ -GT and basolateral  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ , essential enzymes involved in transport function of the enterocyte. We performed experiments to examine the accompanying changes in jejunal antioxidant status during acute acidosis, focusing on the activities of the primary antioxidant enzymes: CAT, SOD, GPx and GR. Enzymatic assays performed on homogenate obtained from everted sac incubated at pH 6, showed significant stimulation of SOD and GR activities, but negligible effects on CAT and GPx activities compared with the control carried out at physiological pH (Figure 2A). In addition, since acidosis has been shown to stimulate free radical formation, which can mediate lipid peroxidation (Fauconneau et al., 1993), we investigated whether peroxidative damage could occur in our experimental conditions. Thus, we measured the level of MDA, a major polyunsaturated fatty acid breakdown product, as an index of lipid peroxidation. Under the acidic experimental conditions set, no significant MDA production was observed compared with the control (Figure 2A).



**Figure 2** Effects of acidosis and  $\text{H}_2\text{O}_2$  on enzymatic activities and lipid peroxidation in mucosal homogenate (A) Effect of acute acidosis on  $\gamma$ -GT,  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ , SOD, CAT, GPx and GR activities and on MDA production of mucosal homogenate after everted sac incubation. White, pH 7.5; black, pH 6.0. (B) Effect of  $\text{H}_2\text{O}_2$  on SOD, CAT, GPx and GR activities of mucosal homogenate after everted sac incubation at two different pHs. White, control; grey,  $\text{H}_2\text{O}_2$ . (C) Effect of  $\text{H}_2\text{O}_2$  on  $\gamma$ -GT and  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$  activities and on MDA production of mucosal homogenate after everted sac incubation at two different pHs. White, control; grey,  $\text{H}_2\text{O}_2$ . Values are means  $\pm$  S.E.M. Number of experiments=4 or 5 with duplicate estimation. \* $P < 0.05$ , \*\* $P < 0.01$ .

### 3.2.2. H<sub>2</sub>O<sub>2</sub> effects on antioxidant enzyme activities of mucosal homogenate after everted sac incubation at two different pHs

It is well known that acidosis markedly potentiates the effects induced by H<sub>2</sub>O<sub>2</sub> exposure, which may also result from impaired antioxidant enzyme functions (Ying et al., 1999). Because of its relatively long half-life and lipophilic nature (Duvall et al., 1998), H<sub>2</sub>O<sub>2</sub> easily crosses cell membranes and enters the cell. So, to determine if acidosis potentiates H<sub>2</sub>O<sub>2</sub> toxicity, we performed experiments by means of everted sac incubation in the presence of 5 mM H<sub>2</sub>O<sub>2</sub> in the mucosal solution, at two different pHs. Literature data suggest that, at this concentration, H<sub>2</sub>O<sub>2</sub> represents a model molecule to induce oxidative stress in various cell types (DuVall et al., 1998; Chatterjee et al., 2000; Song et al., 2005).

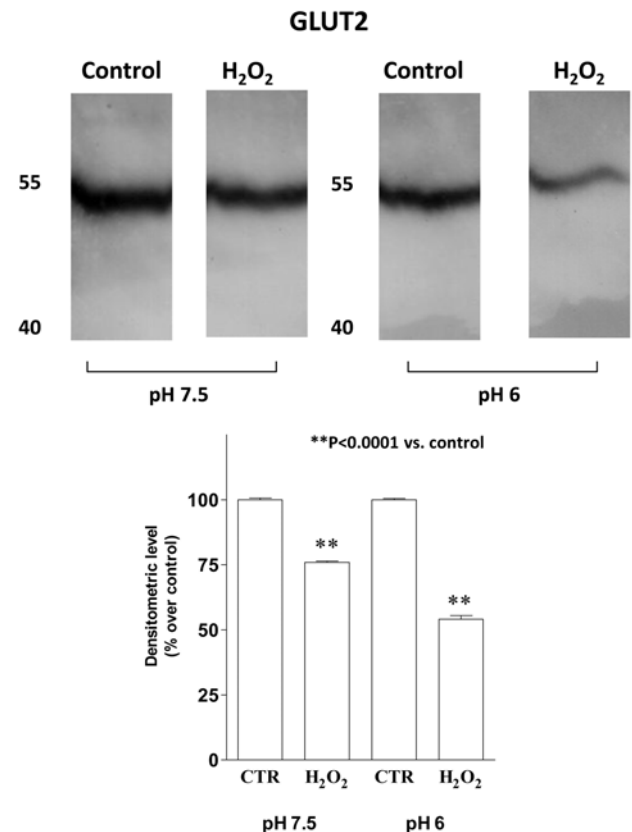
Results give evidence that the antioxidant status of enterocyte is partially influenced by this ROS at both the pHs tested but fails to find positive evidence for acidosis-induced increase of ROS effect. Actually, at both pH values, 5 mM H<sub>2</sub>O<sub>2</sub> induces significant stimulation of both CAT and SOD activities, negligible effects on GPx and GR compared with the control (Figure 2B).

### 3.2.3. H<sub>2</sub>O<sub>2</sub> effects on $\gamma$ -GT and (Na<sup>+</sup>,K<sup>+</sup>)-ATPase activities and MDA production of mucosal homogenate after everted sac incubation at two different pHs

We investigated whether exposure of jejunum to luminal H<sub>2</sub>O<sub>2</sub> could influence the activities of both apical  $\gamma$ -GT and basolateral (Na<sup>+</sup>, K<sup>+</sup>)-ATPase, perhaps with a different toxicity at different pH. We found that, after luminal application of 5 mM H<sub>2</sub>O<sub>2</sub> to the everted sac incubated at normal and acidic pHs, no differences for both the enzymatic activities were obtained with respect to the correspondent controls (Figure 2C). Moreover, we investigated whether exacerbation of lipid peroxidation could occur at acidic pH in the presence of 5 mM H<sub>2</sub>O<sub>2</sub>. As illustrated, it is evident that no significant variation of MDA level was seen (Figure 2C).

### 3.2.4. H<sub>2</sub>O<sub>2</sub> effect on GLUT2 expression in jejunal mucosal homogenate after everted sac incubation at two different pHs

To examine whether some H<sub>2</sub>O<sub>2</sub> effect on the glucose transporter GLUT2 expression in jejunal enterocyte may occur, Western blot analysis was performed on total proteins extracted after everted sac experiments carried out at both physiological and acid pH. Results revealed the presence of a band at 53 kDa (Figure 3), which is the reported molecular weight for GLUT2. Bands obtained from H<sub>2</sub>O<sub>2</sub>-treated tissues are thinner at both pH examined (Figure 3); densitometric analysis of the bands revealed a significant statistical difference in the intensity of the signals with respect to the appropriate control.



**Figure 3** Effect of H<sub>2</sub>O<sub>2</sub> on GLUT2 expression at two different pH values (Upper panels) Western blot analysis for GLUT2 on jejunal total proteins extracted after everted sac experiments. Western blot analysis was carried out on four (control) and four (H<sub>2</sub>O<sub>2</sub> effect) rats at each pH value. (Lower panel) Densitometric analysis of the bands reveals a significant statistical difference in the intensity of the signals between control and H<sub>2</sub>O<sub>2</sub>-treated jejunum.

## 3.3. Induced metabolic acidosis

### 3.3.1. Effect of metabolic acidosis on blood pH and body weight

Rats were made acidotic by addition of 280 mM NH<sub>4</sub>Cl in their drinking water for 7 days; this condition was confirmed by measurements of blood pH immediately before death of treated animals (Table 1). It is evident that the pH value reached by acidotic rats (7.319) is not comparable with the pH set in both isolated membranes and *in vitro* experiments (6.0); however, it is known that in critical illness conditions, pH value can locally fall below 6 (Aranow and Fink, 1996; Barrier et al., 2003).

The acidotic condition was associated with a reduced animal growth: actually, body weight (as a growth index) increases for both the control and acidotic rats during the treatment period; however, the increase was significantly reduced in acidotic rats compared with the controls (Table 2). This result confirms that acidosis is a stressing stimulus, since it is well known that chronic stress induces weight loss (Santos et al., 2000).

**Table 1** Blood pH values after induction of metabolic acidosis

Control	Acidosis
7.390 ± 0.008 (4)	7.319 ± 0.02* (4)

\*  $P < 0.001$ .

### 3.3.2. Effect of metabolic acidosis on $\gamma$ -GT activity in mucosal homogenate

$\gamma$ -GT activity was also examined in mucosal homogenates obtained from the control and acidotic animals. This enzymatic activity remains unaltered in jejunal enterocyte under chronic acidosis condition when compared with the control (Figure 4A).

### 3.3.3. Effect of metabolic acidosis on antioxidant activities and MDA production in mucosal homogenate

Intestinal tissues were harvested from acidotic and control rats and the activities of CAT, SOD, GPx and GR as well as MDA level (a measure of oxidative injury) were determined in mucosal homogenates to focus the influence of the induced metabolic acidosis on these parameters. No significant differences as to the tested enzyme activities and MDA level were observed in acidotic rats compared with the control group in the jejunal tract analysed (Figure 4B).

### 3.3.4. Effect of metabolic acidosis on (Na<sup>+</sup>/K<sup>+</sup>)-ATPase and GLUT2 in mucosal homogenate

To determine whether chronic acidosis induced functional changes in jejunal enterocyte, intestinal tissues were harvested from control and acidotic rats, and (Na<sup>+</sup>, K<sup>+</sup>)-ATPase and GLUT2 protein levels were measured by Western blot analysis. The assays revealed the presence of a band at the specific kDa values, which correspond to the molecular weight for (Na<sup>+</sup>, K<sup>+</sup>)-ATPase alpha 1 subunit (110 kDa, Figure 5A) and GLUT2 (53 kDa, Figure 5B). Densitometric analysis of the bands revealed a significant statistical difference in the intensity of the signals with respect to the appropriate control, for both (Na<sup>+</sup>, K<sup>+</sup>)-ATPase (Figure 5A) and GLUT2 (Figure 5B). (Na<sup>+</sup>, K<sup>+</sup>)-ATPase expression increases, while GLUT2 protein level decreases in acidotic animals (Figures 5A and 5B). (Na<sup>+</sup>, K<sup>+</sup>)-ATPase activity was also measured on mucosal homogenates from control and acidotic rats, confirming the result obtained in Western blot experiments (control: 126.98 ± 13.51 m-units/mg, four experiments; acidosis: 277.17 ± 22.54 m-units/mg, four experiments;  $P < 0.005$ ).

Our studies are relevant here because the observed increased expression and activity of (Na<sup>+</sup>,K<sup>+</sup>)-ATPase and the reduced

expression of GLUT2 quantitative level suggest that, in rat jejunum, a specific regulation of function in response to chronic metabolic acidosis occurs.

## 4. Discussion

We first aimed to investigate the possible effect of acute acidosis on rat jejunal  $\gamma$ -GT activity. Preliminarily, we evaluated the direct action of acidosis on the enzymatic activity by using isolated apical membranes, thus avoiding interactions with other cellular components. This experimental model showed that  $\gamma$ -GT activity is significantly inhibited after incubation of membranes at an acidic pH (Figure 1A), though this result does not imply any correlation between acidosis and ROS action: inhibition could be due either to a direct effect of H<sup>+</sup> ions on the enzyme and/or to some other action on membrane components.

It is well known that acidosis may elicit protective cellular functions as a compensatory response (Song et al., 2005; Valko et al., 2006): subsequently, we used the whole intestinal tract, in which cell and tissue functions are preserved. When rat jejunum was isolated and incubated *in vitro*,  $\gamma$ -GT inhibition by an acidic pH was no longer evident (Figure 2A); in the meantime, SOD and GR activities were enhanced, indicating that acidosis elicited an antioxidant defense. We hypothesize that, in the whole tissue, the increased antioxidant activities are able to contrast acidosis effect observed in isolated membranes. Actually, also in conditions of chronic acidosis,  $\gamma$ -GT activity is unaffected (Figure 4A); however, using the latter experimental model, no variations of antioxidant enzymatic activities are apparent (Figure 4B) at least after 7 days of treatment. Evidence was given that in murine neurons, chronic acidosis reduces the activities of GPx and GR (Ying et al., 1999), while in renal tubular cells, GPx activity is increased (Rustom et al., 2003). The observed differences in chronic stress-induced responses among various tissues may depend on several factors such as metabolic activity rate, susceptibility to oxidants and many more (Kaushik and Kaur, 2003).

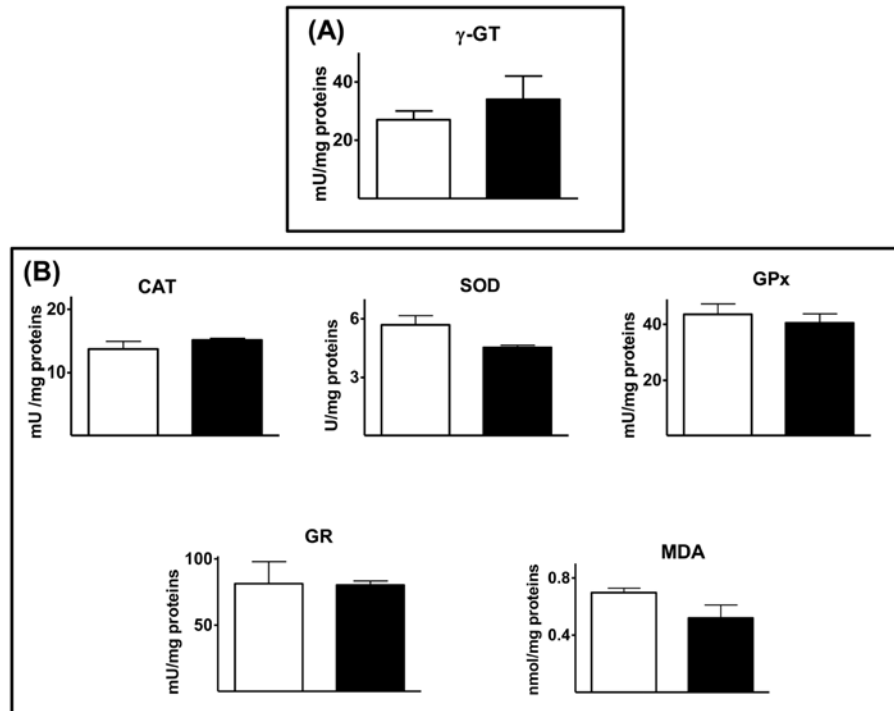
Our results show that neither acute nor chronic acidosis affect lipid peroxidation levels, since there was no MDA production in rat jejunum that could evidence oxidative damage.

To compare the effects of acute and chronic acidosis on some important transport proteins, we chose the (Na<sup>+</sup>,K<sup>+</sup>)-ATPase and GLUT2 on the consideration that clinical studies indicate a greater loss of intestinal barrier function when the extracellular intestinal pH is acidic (Salzman et al., 1994). Our results indicate that acute acidosis does not affect (Na<sup>+</sup>,K<sup>+</sup>)-ATPase activity (Figure 2A), while chronic acidosis significantly enhances both (Na<sup>+</sup>, K<sup>+</sup>)-ATPase activity and protein expression (Figure 5A). An increase in (Na<sup>+</sup>, K<sup>+</sup>)-ATPase mRNA in response to chronic acidosis was reported in rat duodenum (Charoenphandhu et al., 2006). Because chronic acidosis is a systemic condition, and several organs would act in concert to reduce the detrimental consequences (Wongdee et al., 2009), the observed enhanced jejunal (Na<sup>+</sup>, K<sup>+</sup>)-ATPase could be part of the body response that may involve adaptive changes at both molecular and organ levels.

**Table 2** Rat weight variations after induction of metabolic acidosis

Control (g)	Acidosis (g)
+58.2 ± 2.4 (4)	+39.9 ± 4.9* (4)

\*  $P < 0.001$ .



**Figure 4** Metabolic acidosis effect on  $\gamma$ -GT and oxidative stress parameters

(A)  $\gamma$ -GT activity of mucosal homogenate after induction of metabolic acidosis. White, control; black, acidosis. (B) SOD, CAT, GPx, GR activities and MDA production of mucosal homogenate after induction of metabolic acidosis. White, control; black, acidosis. Values are means  $\pm$  S.E.M. Number of experiments=4 with duplicate estimation.

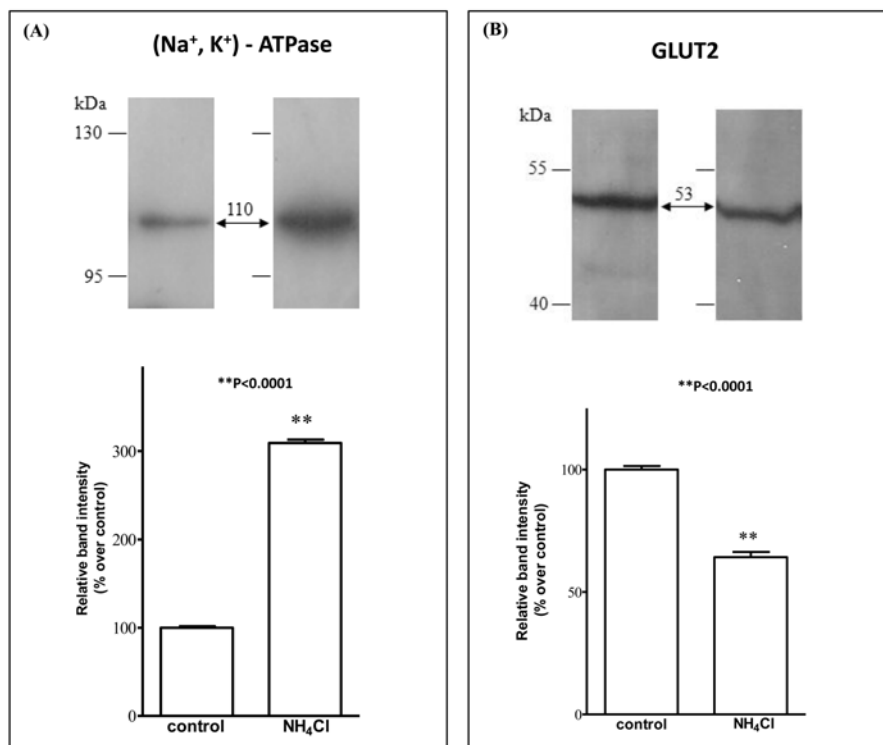
Western blot experiments performed to evaluate GLUT2 expression give evidence for a significant reduction both in acute (Figure 3) and chronic (Figure 5B) acidosis. Moreover, the short time required for the down-regulation in acute acidosis suggests that regulation occurs at the posttranslational level. This point needs further investigation to clarify whether the reduced GLUT2 expression could be ascribed to the stressing condition induced by acidosis. The various ROS can exert different effects according to their nature and concentration. Using isolated membranes, in the presence of peroxidase inhibitors,  $\gamma$ -GT appears to be insensitive to all the tested ROS at both pH values, with the exception of  $H_2O_2$  at millimolar concentrations (Figures 1B and 1C). Owing to the important role of  $\gamma$ -GT in GSH homeostasis, its resistance to ROS might preserve the cellular antioxidant power in oxidative stress conditions, thus avoiding pathological effects. The mechanism responsible for  $\gamma$ -GT inhibition by  $H_2O_2$  in isolated membranes is unclear, since, besides producing direct effects on enzymatic proteins, ROS may cause alterations in lipid composition and in membrane fluidity, thus indirectly modulating the enzymatic activity.

Using the whole isolated rat jejunum, the effects of  $H_2O_2$  (whose concentration was monitored and adjusted during the experiment) were examined in terms of perturbations in antioxidant defense.  $H_2O_2$  was chosen as oxidant agent because it is a precursor of more potent radical species, and it was the only one influencing  $\gamma$ -GT activity in isolated membranes (Figure 1B). Previous experiments carried out with isolated rat jejunum (Orsenigo et al., 2007) demonstrated that, in this experimental condition,  $H_2O_2$  does not

cause mucosal injury. Results reported show that, in the presence of  $H_2O_2$ , CAT and SOD activities are enhanced (Figure 2B). It is known that SOD catalyses the conversion of  $O_2^{\cdot -}$  into  $H_2O_2$  and  $O_2$ .  $H_2O_2$  is scavenged at higher concentrations by CAT and at lower concentrations by GPx. The relative contributions of CAT and GPx in the decomposition of  $H_2O_2$  follow tissue specificity (Kaushik and Kaur, 2003): our results suggest that in rat jejunum, CAT is mainly involved in this process.

When  $\gamma$ -GT activity was also assayed in the same experimental condition (Figure 2C), the inhibitory effect of  $H_2O_2$  observed in isolated membranes (Figure 1B) was no longer evident, suggesting that the enzyme activity was preserved by the antioxidant defense induced by  $H_2O_2$ . Actually, injuries caused by increased ROS are not evident unless the potency of antioxidative defense is exhausted (Augustin et al., 1997).  $(Na^+, K^+)$ -ATPase activity is unaffected by  $H_2O_2$  either (Figure 2C). Interestingly, in a previous research (Orsenigo et al., 2007), we demonstrated that in the presence of inhibitors of antioxidant activities,  $(Na^+, K^+)$ -ATPase was inhibited after exposure to  $H_2O_2$ . This observation suggests that, as for  $\gamma$ -GT, in the present research, the increased CAT and SOD activities did not allow  $H_2O_2$  inhibitory effect.

Results obtained so far indicate that, for all the tested enzyme activities, the pattern of responses to oxidant agents is not affected by acidosis. In general, the jejunal tract of intestine seems quite resistant to acidosis conditions; actually, an acid microclimate overlying enterocyte brush border is always present in jejunum (Thwaites and Anderson, 2007). Intestinal cells could adapt to



**Figure 5** Metabolic acidosis effect on  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$  and GLUT2 expression  
Western blot analysis for (A)  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$  (subunit alpha 1), (B) GLUT2, on jejunum total proteins extracted after induction of metabolic acidosis. In both cases, Western blot analysis was carried out on four control and four acidotic rats. Densitometric analyses of the bands reveal significant statistical differences in the intensities of the signals.

acidosis by increasing their individual buffer capacity, suggesting a specific regulation of function in response to these conditions.

## 5. Conclusion

This study elucidates the relationship between acidosis and oxidative parameters in the jejunum. Inhibition of  $\gamma\text{-GT}$  activity caused by both acidic pH and  $\text{H}_2\text{O}_2$  in isolated membranes disappears using the whole tissue. In the isolated jejunum, acute acidosis and  $\text{H}_2\text{O}_2$  evoke a defense by antioxidant enzymes, whereas after 7 days of chronic acidosis, the compensatory response might be accomplished; however, effects on  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$  and GLUT2 expressions are evident. The information reported herein might be useful to define therapeutic strategies in order to ameliorate intestinal responses to acidosis associated with gastrointestinal diseases.

### Author contribution

All authors contributed equally to this work.

### Funding

This research was supported by Ministero Dell' Istruzione, Dell' Università e Della Ricerca, Italy.

## References

- Aebi HE. Catalase. In: Bergmeyer HU, editor. Methods in enzymatic analysis. Weinheim: Verlag Chemie; 1983. p. 278–82.
- Alva N, Carbonell T, Palomeque J. Hypothermic protection in an acute hypoxia model in rats: acid-base and oxidant/antioxidant profiles. *Resuscitation* 2010;81:609–16.
- Antonova OA, Loktionova SA, Romanov YA, Shustova ON, Khachikian MV, Mazurov A. Activation and damage of endothelial cells upon hypoxia/reoxygenation. Effect of extracellular pH. *Biochemistry* 2009;74:605–12.
- Anwer T, Sharm M, Pillai KK, Haque SE, Alam MM, Zaman MS. Protective effect of bezafibrate on streptozomicin-induced oxidative stress and toxicity in rats. *Toxicol* 2007;1229:165–72.
- Aranow JS, Fink MP. Determinants of intestinal barrier failure in critical illness. *Brit J Anaesth* 1996;77:71–81.
- Augustin W, Wiswedel I, Noack H, Reinheckel T, Reichelt O. Role of endogenous antioxidants in defense against functional damage and lipid peroxidation in rat liver mitochondria. *Mol Cell Biochem* 1997;174:199–205.
- Barrier L, Barc S, Fauconneau B, Pontcharraud R, Kelani A, Bestel E, Page G. Evidence that acidosis alters the high-affinity dopamine uptake in rat striatal slices and synaptosomes by different mechanisms partially related to oxidative damage. *Neurochem Int* 2003;42:27–34.
- Bradford MN. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal Biochem* 1976;72:248–54.
- Charoenphandhu N, Tudpor K, Pulsook N, Krishnamra N. Chronic metabolic acidosis stimulated transcellular and solvent drag-induced calcium transport in the duodenum of female rats. *Am J Physiol Gastrointest Liver Physiol* 2006;291:G446–55.

- Chatterjee PK, Cuzzocrea S, Brown PAJ, Zacharowski K, Stewart KN, Mota-Filipe H, Thiemermann C. Tempol, a membrane-permeable radical scavenger, reduces oxidant stress-mediated renal dysfunction and injury in the rat. *Kidney Int* 2000;58:658–73.
- Cutaia M, Kroczyński J, Tollefson K. pH-dependent oxidant production following inhibition of the mitochondrial electron transport chain in pulmonary endothelial cells. *Endothelium* 2002;9:109–21.
- Dringen R, Hamprecht B. Involvement of glutathione peroxidase and catalase in the disposal of exogenous hydrogen peroxide by cultured astroglial cells. *Brain Res* 1997;759:67–75.
- Duvall MD, Guo YI, Matalon S. Hydrogen peroxide inhibits cAMP-induced  $\text{Cl}^-$  secretion across colonic epithelial cells. *Am J Physiol* 1998;275:C1313–22.
- Faelli A, Burlini N, Esposito G, Tosco M, Capraro V. The rat and hamster jejunum during transintestinal transport *in vitro*. *Arch Int Physiol Biochem* 1979;87:73–86.
- Fauconneau B, Tallineau C, Huguet F, Guillard O, Piriou A. Iron- and lactic acid-induced lipid peroxidation in rat kidney homogenates and slices. *Biochem Mol Biol Int* 1993;31:421–7.
- Guzik TJ, Olszanecki R, Sadowski J, Kapelak B, Rudzinski P, Jopek A et al. Superoxide dismutase activity and expression in human venous and arterial bypass graft vessels. *J Physiol Pharmacol* 2005;56:313–23.
- Halliwell B, Gutteridge JMC. *Free radicals in biology and medicine*. Oxford: Clarendon Press; 1999.
- Hassan W, Ibrahim M, Deobald AM, Braga AL, Nogueira CW, Rocha JB. pH-dependent Fe (II) pathophysiology and protective effect of an organoselenium compound. *FEBS Lett* 2009;583:1011–6.
- Hazell AS. Astrocytes are a major target in thiamine deficiency and Wernicke's encephalopathy. *Neurochem Int* 2009;55:129–35.
- Homma H, Hoy E, Xu DZ, Lu Q, Feinman R, Deitch E. The female intestine is more resistant than the male intestine to gut injury and inflammation when subjected to conditions associated with shock states. *Am J Physiol Gastrointest Liver Physiol* 2005;288:G466–72.
- Kaushik S, Kaur J. Chronic cold exposure affects the antioxidant defense system in various rat tissues. *Clin Chim Acta* 2003;333:69–77.
- Klassen NV, Marchington D, McGowan HCE.  $\text{H}_2\text{O}_2$  determination by the  $\text{I}_3^-$  method and by  $\text{KMnO}_4$  titration. *Anal Chem* 1994;66:2921–5.
- Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979;95:351–8.
- Ojano-Dirain C, Iqbal M, Wing T, Cooper M, Bottje W. Glutathione and respiratory chain complex activity in duodenal mitochondria of broilers with low and high feed efficiency. *Poultry Sci* 2005;84:782–8.
- Orsenigo MN, Faelli A, Porta C, Sironi C, Laforenza U, Paulmichl M and Tosco M. Oxidative stress reduces transintestinal transports and ( $\text{Na}^+$ ,  $\text{K}^+$ )-ATPase activity in rat jejunum. *Arch Biochem Biophys* 2007;466:300–7.
- Orsenigo MN, Tosco M, Esposito G, Faelli A. The basolateral membrane of rat enterocyte: its purification from brush border contamination. *Anal Biochem* 1985;144:577–83.
- Pedoto A, Nandi J, Oler A, Camporesi EM, Hakim TS, Levine RA. Role of acid nitric oxide in acidosis induced intestinal injury in anaesthetized rats. *J Lab Clin Med* 2001;138:270–6.
- Prabhu R, Anup R, Balasubramanian KA. Surgical stress induces phospholipid degradation in the intestinal brush border membrane. *J Surg Res* 2000;94:178–84.
- Prabhu R, Balasubramanian KA. Effect of oxidants on small intestinal brush border membranes and colonic apical membranes – a comparative study. *Comp Biochem Physiol C Toxicol Pharmacol* 2003;134:329–39.
- Rahman I, Biswas SK, Kirkham PA. Regulation of inflammation and redox signaling by dietary polyphenols. *Biochem Pharmacol* 2006;72:1439–52.
- Rustom R, Wang B, McArdle F, Shalamanova L, Alexander J, McArdle A et al. Oxidative stress in a novel model of chronic acidosis in LLC-PK1 cells. *Nephron Exp Nephrol* 2003;95:13–23.
- Salzman AL, Wang H, Wollet PS, Fink MP. Endotoxin-induced ileal mucosal hyperpermeability in pigs: role of tissue acidosis. *Am J Physiol Gastrointest Liver Physiol* 1994;266:G633–46.
- Santos J, Benjamin M, Yang PC, Prior T, Perdue MH. Chronic stress impairs rat growth and jejunal epithelial barrier function: role of mast cells. *Am J Physiol Gastrointest Liver Physiol* 2000;278:G847–54.
- Song HJ, Lee TS, Jeong JH, Min YS, Shin CY, Sohn UD. Hydrogen peroxide-induced extracellular signal-regulated kinase activation in cultured feline ileal smooth muscle cells. *J Pharmacol Exp Ther* 2005;312:391–8.
- Thwaites DT, Anderson CM.  $\text{H}^+$ -coupled nutrient, micronutrient and drug transporters in the mammalian small intestine. *Exp Physiol* 2007;92:603–19.
- Tosco M, Faelli A, Sironi C, Gastaldi G, Orsenigo MN. A creatine transporter is operative at the brush border level of rat jejunal enterocyte. *J Membrane Biol* 2004;202:85–95.
- Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact* 2006;160:1–40.
- Wongdee K, Teerapornpuntakit J, Riengrojpitak S, Krishnamra N, Charoenphandhu N. Gene expression profile of duodenal epithelial cells in response to chronic metabolic acidosis. *Mol Cell Biochem* 2009;321:173–188.
- Ying W, Han SK, Miller JW, Swanson RA. Acidosis potentiates oxidative neuronal death by multiple mechanism. *J Neurochem* 1999;73:1549–56.

Received 3 June 2010/22 November 2010; accepted 14 December 2010

Published as Immediate Publication 14 December 2010, doi 10.1042/CBI20100428