

## Oxidative stress, antioxidants and intestinal calcium absorption

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**Author contributions:** Diaz de Barboza G, Guizzardi S, Moine L and Tolosa de Talamoni N participated in information collection, analysis, information organization, writing, figure design, and final editing.

**Supported by** Dr. Nori Tolosa de Talamoni from CONICET, No. PIP 2013-2015 and No. SECYT (UNC) 2016, Argentina.

**Conflict-of-interest statement:** No conflicts of interest, financial or otherwise, are declared by the authors.

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**Manuscript source:** Invited manuscript

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**Received:** January 7, 2017

**Peer-review started:** January 9, 2017

**First decision:** February 9, 2017

**Revised:** March 1, 2017

**Accepted:** March 30, 2017

**Article in press:** March 30, 2017

**Published online:** April 28, 2017

### Abstract

The disequilibrium between the production of reactive oxygen (ROS) and nitrogen (RNS) species and their elimination by protective mechanisms leads to oxidative stress. Mitochondria are the main source of ROS as by-products of electron transport chain. Most of the time the intestine responds adequately against the oxidative stress, but with aging or under conditions that exacerbate the ROS and/or RNS production, the defenses are not enough and contribute to developing intestinal pathologies. The endogenous antioxidant defense system in gut includes glutathione (GSH) and GSH-dependent enzymes as major components. When the ROS and/or RNS production is exacerbated, oxidative stress occurs and the intestinal  $\text{Ca}^{2+}$  absorption is inhibited. GSH depleting drugs such as DL-buthionine-S,R-sulfoximine, menadione and sodium deoxycholate inhibit the  $\text{Ca}^{2+}$  transport from lumen to blood by alteration in the protein expression and/or activity of molecules involved in the  $\text{Ca}^{2+}$  transcellular and paracellular pathways through mechanisms of oxidative stress, apoptosis and/or autophagy. Quercetin, melatonin, lithocholic and ursodeoxycholic acids block the effect of those drugs in experimental animals by their antioxidant, anti-apoptotic and/or anti-autophagic properties. Therefore, they may become drugs of choice for treatment of deteriorated intestinal  $\text{Ca}^{2+}$  absorption under oxidant conditions such as aging, diabetes, gut inflammation and other intestinal disorders.

**Key words:** Transcellular and paracellular  $\text{Ca}^{2+}$  pathways; DL-buthionine-S,R-sulfoximine; Menadione; Sodium deoxycholate; Lithocholic acid; Ursodeoxycholic acid; Melatonin

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**Core tip:** Gut includes glutathione depleting drugs inhibit the intestinal  $\text{Ca}^{2+}$  absorption by alteration in the

protein expression and/or activity of molecules involved in the transcellular and paracellular  $\text{Ca}^{2+}$  pathways through mechanisms of oxidative stress, apoptosis and/or autophagy. Quercetin, melatonin, lithocholic and ursodeoxycholic acids block the effect of those drugs in experimental animals by their antioxidant, anti-apoptotic and anti-autophagic properties. Therefore, they may become drugs of choice for treatment of deteriorated intestinal  $\text{Ca}^{2+}$  absorption under oxidant conditions such as aging, diabetes, gut inflammation and other intestinal disorders.

Diaz de Barboza G, Guizzardi S, Moine L, Tolosa de Talamoni N. Oxidative stress, antioxidants and intestinal calcium absorption. *World J Gastroenterol* 2017; 23(16): 2841-2853 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i16/2841.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i16.2841>

## INTRODUCTION

The imbalance between the production of reactive oxygen (ROS) and nitrogen (RNS) species and their elimination by protective mechanisms leads to oxidative stress<sup>[1]</sup>. This response occurs in various pathophysiological conditions such as aging, inflammation, cardiovascular and neurodegenerative diseases, damaging many components including proteins, DNA/RNA and lipids<sup>[2-5]</sup>. The cellular dysfunctions caused by excessive ROS and/or RNS might produce loss of energy metabolism, altered cell signaling and cell cycle, gene mutations and impaired cellular transport mechanisms. Taken together, the oxidative stress promotes decreased biological activities, immune activation and inflammation. Moreover, the nutritional stress produced by high-fat and high-carbohydrate diets also generates oxidative stress, which leads to initiation of pathogenic milieu and development of different chronic diseases<sup>[6-8]</sup>. ROS are also generated by other exogenous sources such as ultraviolet radiation, alcohol consumption, cigarette smoking, ingestion of nonsteroidal anti-inflammatory drugs and infections<sup>[9,10]</sup>. Ischemia/reperfusion (I/R) injuries also contribute to exacerbating ROS production<sup>[11]</sup>. ROS are normally produced within the body in small quantities and are involved in the regulation of processes, maintenance of cell homeostasis and functions such as signal transduction, gene expression, and activation of receptors<sup>[12]</sup>. Mitochondria are one of the most relevant sources of ROS and RNS. The organelles produce ROS and organic peroxides as by-products during the functioning of the electron transport chain (ETC), and, in hypoxic conditions, they also produce nitric oxide ( $\cdot\text{NO}$ ), one RNS that can further lead to produce reactive aldehydes, malondialdehyde and 4-hydroxynonenal<sup>[13,14]</sup>. Peroxisomes also play a major role in the cellular ROS and RNS-metabolism, not

only because they contain a large number of ROS-producing enzymes, but they also interplay with other organelles, mainly with the mitochondria and endoplasmic reticulum (ER)<sup>[15]</sup>. The accumulation of unfolded and misfolded proteins in the ER lumen, known as ER stress, activates the unfolded protein response, which enhances the ER capacity for protein folding and modification, attenuates global mRNA translation, and disposes misfolded proteins by ER-associated protein degradation and autophagy. The dysregulated disulfide bond formation and breakage in a stressed ER, may produce ROS accumulation and cause oxidative stress<sup>[16]</sup>.

The small intestine is the main organ of exposure and/or absorption of nutrients, toxic food contaminants and therapeutic drugs. It is also exposed to secreted metabolites and the metabolic products coming from the intestinal bacteria. The alteration of the integrity and/or function of the intestinal epithelium produce a negative impact on the rest of the organism<sup>[17]</sup>. Therefore, the disequilibrium in the redox state of gut is not only important for its functionality, but also for the entire body. Fortunately, most of the time the intestine responds adequately against the oxidative stress, but with aging or under conditions that exacerbate the ROS and/or RNS production the defenses are not enough, which contribute to developing intestinal pathologies such as inflammatory bowel disease (IBD), gastroduodenal ulcers, colon cancer and others<sup>[18-20]</sup>.

## FORMATION OF ROS AND RNS IN THE GUT

ROS are not only highly reactive and continuously produced as by-products of cellular respiration, but are also generated by enzymatic reactions. ROS include radical compounds such as superoxide ( $\text{O}_2^{\cdot-}$ ), hydroxyl radicals ( $\cdot\text{HO}$ ), lipid hydroperoxides, and reactive nonradical compounds including singlet oxygen ( $^1\text{O}_2$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), hypochlorous acid ( $\text{HOCl}$ ) and others<sup>[21]</sup>. RNS include radical compounds such as  $\cdot\text{NO}$ , nitrogen dioxide ( $\cdot\text{NO}_2$ ), and nonradical compounds such as peroxynitrite ( $\text{ONOO}^{\cdot-}$ ) and dinitrogen trioxide ( $\text{N}_2\text{O}_3$ ). Most of these compounds are unstable because of unpaired electrons in the outer electron orbit. When ROS are accumulated, the major cellular antioxidants such as glutathione (GSH) and thioredoxin alter their redox state, and the antioxidant defenses decline.

In the mitochondria, the electron leakage from ETC complexes I and III produces a reduction of molecular oxygen forming  $\text{O}_2^{\cdot-}$ <sup>[22]</sup>. In contrast, cytochrome c oxidase (complex IV) is not an important source of ROS. It reduces molecular oxygen to two molecules of  $\text{H}_2\text{O}$  through a four-electron reduction<sup>[23]</sup>. NADPH oxidase, present in the plasma membrane and phagosomes of phagocytes (monocytes, macrophages,

neutrophils and eosinophils), also produces an important amount of O<sub>2</sub><sup>•-</sup> in the intestine, mainly in conditions of inflammation induced by *Helicobacter pylori*, IBD, and tumor development<sup>[8]</sup>. Xanthine oxidase (XO) is another enzyme that generates O<sub>2</sub><sup>•-</sup> as a by-product by oxidation of hypoxanthine to xanthine, and then to uric acid during purine catabolism. In the I/R of gut, this enzyme produces an enormous quantity of ROS because the oxidation of hypoxanthine is increased<sup>[24,25]</sup>. XO exists mainly in the small intestine and it may be a major source of ROS in patients during colon surgery<sup>[26]</sup>. The enzyme is predominantly present as xanthine dehydrogenase under physiological conditions, but it can be transformed by proteolysis into XO. In acute pancreatitis, XO is mobilized from the gastrointestinal endothelial cell surface<sup>[27]</sup>. The enzymes of the XO family share a molybdenum cofactor (Moco), which is a trace element and crucial for life<sup>[28]</sup>. The reason the mature enterocytes, located at the tip of the microvilli, are more sensitive to I/R than their undifferentiated counterparts located in the villus base seems to be related, at least in part, to the higher expression and activity of XO<sup>[29]</sup>. The nutritional deficiency in Mo has been associated with high risk of esophageal cancer in populations consuming food grown in molybdenum-poor soil<sup>[30]</sup>. ROS are also produced in the intestine by other enzymes such as myeloperoxidases, lipoxygenases, cyclooxygenases and transition metals as copper and iron. •NO is a weak oxidant generated by oxidation of L-arginine, reaction catalyzed by nitric oxide synthase (NOS). When •NO combines with O<sub>2</sub><sup>•-</sup>, it generates OONO<sup>-</sup>, which is highly reactive<sup>[31]</sup>. OONO<sup>-</sup> provokes enterocyte apoptosis, reduces enterocyte proliferation and interferes with epithelial renewal<sup>[32]</sup>. •NO and OONO<sup>-</sup> produce stable nitrite and nitrate ions, which can be accumulated in cells leading to form high reactive intermediates, such as •NO<sub>2</sub> and N<sub>2</sub>O<sub>3</sub>. These intermediates may cause nitration and nitrosation of DNA, RNA, proteins and lipids with the consequent dysfunction of these molecules<sup>[33]</sup>.

## ENDOGENOUS ANTIOXIDANT DEFENSE SYSTEM IN THE GUT

Any substance or compound that scavenges oxygen free radicals or inhibits the cellular oxidation process is considered an antioxidant<sup>[34]</sup>. The main non enzymatic antioxidants in gut are GSH and the thioredoxin system. GSH is a tripeptide formed by L-glutamate, L-glycine and L-cysteine, and is present in millimolar concentration (2-10 mmol/L) in all eukaryotic cells. The oxidation of GSH to disulfide of glutathione (GSSG) and subsequent decrease in the GSH/GSSG couple is often a useful indicator of cellular oxidative stress<sup>[35]</sup>. There are different pools of GSH in the cell. The total cellular GSH/GSSG ratio mainly represents the cytoplasmic GSH/GSSG pool. GSH/GSSG ratios

are not in equilibrium with each other in mitochondria, nucleus, the secretory pathway and the extracellular space<sup>[36]</sup>. Mitochondrial GSH is responsible for 15% to 30% of total GSH<sup>[37]</sup>. In the ER, the GSH/GSSG ratio ranges between 3/1 and 1/1. It is more oxidized than cytoplasmic GSH/GSSG ratio, which varies between 30/1 to 100/1. GSH in the ER was mainly detected as protein mixed disulfides, which means that it would regulate the activity of redox-active thiol-containing proteins<sup>[38]</sup>. Protein S-glutathionylation is independently controlled in the cytoplasmic and nuclear compartments and the GSH/GSSG redox potential is probably more reduced in nucleus than in cytoplasm<sup>[36]</sup>. The cytosolic enzymes  $\gamma$ -glutamylcysteine ligase and GSH synthetase are involved in *de novo* GSH synthesis, while the regeneration of GSH from GSSG is catalyzed by NADPH-dependent GSSG reductase<sup>[39]</sup>. In transport epithelial cells as occurs in enterocytes,  $\gamma$ -glutamyltransferase and dipeptidase catalyze the hydrolysis of extracellular GSH to its constituent amino acids<sup>[40]</sup>.

The distribution between nuclear GSH and cytoplasmic GSH is dynamic. The GSH concentration in nucleus is 4 times higher than in cytoplasm during cell cycle and is equal in both compartments when cells are confluent<sup>[41]</sup>. The intestinal GSH levels depend on the *de novo* synthesis, regeneration from GSSG and the GSH uptake at the apical membrane<sup>[40]</sup>. It appears that the cellular GSH/GSSG redox status governs cell transitions from quiescence to that of a proliferative state, as well as the growth arrest, differentiation and apoptosis, not only in the intestine but also in other cells. A reducing redox environment favors proliferation, whereas an oxidized milieu stimulates growth arrest and differentiation<sup>[42]</sup>. If the redox environment is highly oxidized, it promotes apoptosis or necrosis. Mitochondria are involved in the oxidant-mediated cellular apoptosis<sup>[43]</sup>. Loss of mitochondrial GSH (mtGSH) produces mitochondrial transition pore opening<sup>[44]</sup>, inhibits ETC, decreases ATP and increases ROS generation, which leads to cell apoptosis<sup>[45,46]</sup>. mtGSH also preserves intestinal mitochondrial genes and functional integrity<sup>[47]</sup>. Another GSH pool, the luminal GSH pool, has an important role in the processes of absorption and detoxification as well as in maintenance of mucus fluidity<sup>[48-50]</sup>.

The thioredoxin system is composed of thioredoxin (Trx) and thioredoxin reductases (TrxR). It has a large number of functions in DNA synthesis, defense against oxidative stress and apoptosis or redox signaling. It is located in the cytoplasm, membranes, mitochondria, and in the extracellular space. Oxidized Trxs are reactivated by TrxRs through the reducing power of NADPH<sup>[51]</sup>. Trx expression is very high in the intestine and has an important role in gut immune response<sup>[52]</sup>. It has been demonstrated that Trx is involved in redox regulation of human  $\beta$ -defensin 1, a protein with antimicrobial activity<sup>[53]</sup>. Ulcerative colitis involving Trx

as a candidate marker has been revealed by proteomic profiles of colonic biopsies<sup>[54]</sup>.

The major GSH-dependent enzymatic antioxidants in the intestine are superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-reductase (GR) and catalase (CAT). SOD and CAT provide major antioxidant defenses against ROS<sup>[8]</sup>. SODs are enzymes that catalyze dismutation of  $\text{O}_2^{\cdot -}$  into  $\text{O}_2$  and  $\text{H}_2\text{O}_2$ . In humans there are three isoforms of SOD: cytosolic copper and zinc-containing enzyme ( $\text{Cu}^{2+}/\text{Zn}^{2+}$ -SOD), manganese-requiring mitochondrial enzyme ( $\text{Mn}^{2+}$ -SOD), and an extracellular  $\text{Cu}^{2+}/\text{Zn}^{2+}$  containing SOD.  $\text{Mn}^{2+}$ -SOD has an indispensable role in protecting aerobic life from deleterious effects of oxygen. It could be considered as the guardian of the powerhouse.  $\text{Mn}^{2+}$ -SOD can scavenge  $\text{O}_2^{\cdot -}$  generated by the ETC complexes and may be important in preventing ROS-induced inactivation of these complexes<sup>[55]</sup>. Injuries of the GIT can be prevented by SOD in the gastrointestinal mucosa. Intestinal epithelia from IBD patients have enhanced levels of all three SOD isoforms<sup>[56]</sup>.  $\text{H}_2\text{O}_2$ , itself a ROS, is decomposed into water by different enzymes including GPx, CAT and peroxiredoxins<sup>[57]</sup>. GPx reduces not only  $\text{H}_2\text{O}_2$ , but also lipid hydroperoxides. In the intestine there are four isoforms of GPx<sup>[58]</sup>. GPx1 is present in all cell types of the gut, GPx2 is predominantly expressed in the epithelial cells, GPx3 is secreted in plasma, and GPx4 is expressed in epithelial cells and the lamina propria<sup>[59]</sup>. GPx2 is in the first line of defense against ROS derived from inflammation associated with both pathogenic and nonpathogenic bacteria from the intestine<sup>[58]</sup>. GR is a ubiquitous enzyme that reduces GSSG to GSH. GR is a NADPH-dependent flavoprotein. Two electrons of reducing power are extracted from NADPH and transferred to reducing GSSG into two molecules of GSH<sup>[60]</sup>. CAT, which is found mainly in peroxisomes, dismutates  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$  and  $\text{O}_2$ . It is present in all human organs and in many pathogens in the GIT to evade host response and survive within the host. In addition, CAT is also expressed in mitochondria and is considered to protect cells from apoptosis<sup>[61]</sup>. Taken together, all these enzymes and endogenous non enzymatic antioxidants contribute to the equilibrium in the redox state of the intestine under physiological conditions. However, excessive ROS and/or RNS may still lead to oxidative damage to tissue and organs. Hence, the application of antioxidants seems to be a rational therapeutic strategy to prevent or cure diseases involving oxidative stress.

## MECHANISMS OF INTESTINAL CALCIUM ABSORPTION

The intestinal  $\text{Ca}^{2+}$  absorption is an active process (ATP dependent) that mainly occurs in the small intestine, which is responsible for approximately 90% of overall  $\text{Ca}^{2+}$  absorption<sup>[62]</sup>. The sojourn time in each intestinal

segment and the  $\text{Ca}^{2+}$  solubility are important factors affecting the intestinal  $\text{Ca}^{2+}$  absorption. The solubility depends on the environmental pH. Although the solubility is higher in the stomach because of the acidic pH, this is less relevant because  $\text{Ca}^{2+}$  is absorbed in the small and large intestine. It appears that the duodenum is the site with the maximum solubility of  $\text{Ca}^{2+}$  because the average pH is 6.0, the lowest of the entire gut<sup>[63]</sup>. The  $\text{Ca}^{2+}$  absorption rate occurs in this order: duodenum > jejunum > ileum<sup>[64]</sup>. Since the major source of ATP is the mitochondria, the integrity and functionality of these organelles are necessary to produce an appropriate intestinal  $\text{Ca}^{2+}$  absorption. Growth, pregnancy and lactation promote the intestinal  $\text{Ca}^{2+}$  absorption, while aging decreases cation absorption<sup>[65-68]</sup>. The efficiency of the intestine to absorb  $\text{Ca}^{2+}$  increases not only when the requirements enhance, but also when the intake is low<sup>[69]</sup>. In other words, the intestinal  $\text{Ca}^{2+}$  absorption depends on the physiological needs of  $\text{Ca}^{2+}$ .

There are two mechanisms of intestinal  $\text{Ca}^{2+}$  absorption: transcellular and paracellular. Both mechanisms are regulated by hormones, nutrients and other factors. The transcellular pathway comprises three steps: entry across the brush border membrane (BBM) of the enterocytes, intracellular diffusion from one pole to the other of the epithelial cells and exit through the basolateral membrane (BLM). In the BBM there are  $\text{Ca}^{2+}$  epithelial channels, called transient receptor potential vanilloid-family member 6 (TRPV6) and transient receptor potential vanilloid-family member 5 (TRPV5), which are apparently involved in the  $\text{Ca}^{2+}$  uptake from the lumen to inside the cell through the BBM<sup>[70]</sup>. TRPV6 predominates in the intestine, whereas TRPV5 in the kidney. Cav1.3 is another channel from the BBM, which is apparently involved in the active transcellular  $\text{Ca}^{2+}$  absorption. TRPV6 would predominate under polarizing conditions between meals, whereas Cav1.3 would play a dominant role under depolarizing conditions during digestion, mainly when diet is plentiful in  $\text{Ca}^{2+}$ <sup>[71]</sup>. In contrast, some authors demonstrate that Cav1.3 is not critical for active intestinal  $\text{Ca}^{2+}$  absorption *in vivo* in mice<sup>[72]</sup>. Calbindin D<sub>9k</sub> in mammals and calbindin D<sub>28k</sub> in birds are cytoplasmic proteins that carry the cations as a ferry from the BBM to the BLM<sup>[73]</sup>. Calbindins also buffer  $\text{Ca}^{2+}$ , which maintains intracellular  $\text{Ca}^{2+}$  concentrations below  $10^{-7}$  M and prevents cell death by apoptosis. The excess of  $\text{Ca}^{2+}$  that occurs when there is a down-regulation of calbindins may trigger apoptosis in the epithelial cells, as shown in different tissues<sup>[74,75]</sup>. In the BLM, there are two proteins involved in the  $\text{Ca}^{2+}$  exit to the lamina propria: the plasma membrane  $\text{Ca}^{2+}$ -ATPase (PMCA), an ATP-dependent transporter that pumps  $\text{Ca}^{2+}$  out of the cytosol<sup>[76]</sup>, and the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX), whose activity depends on the gradient created by  $\text{Na}^+/\text{K}^+$ -ATPase<sup>[77]</sup>. The predominant isoform in the intestine



is PMCA<sub>1b</sub>, mainly located in the caveolae. PMCA<sub>1b</sub> is responsible for the major Ca<sup>2+</sup> exit to the lamina propria. Its expression and activity are higher in villus tip enterocytes than in villus crypt cells, which is in agreement with the hypothesis that mature enterocytes have the greatest ability for transcellular Ca<sup>2+</sup> movement<sup>[78]</sup>. NCX also presents several isoforms, but NCX1 predominates in the intestine<sup>[79]</sup>. It has a stoichiometry of 3 Na<sup>+</sup>:1 Ca<sup>2+</sup> and can function in either a forward mode (Ca<sup>2+</sup> extrusion) or in a reversed mode (Ca<sup>2+</sup> entry), depending on the Na<sup>+</sup> and Ca<sup>2+</sup> gradients and the membrane potential<sup>[80]</sup>. Another novel protein 4.1R, which co-localizes with PMCA<sub>1b</sub>, could have an important role in the transcellular Ca<sup>2+</sup> pathway, but its physiological function is not well known<sup>[81]</sup>.

The paracellular Ca<sup>2+</sup> pathway occurs through tight junctions (TJ), intercellular structures where plasma membranes of adjacent enterocytes have very close contact. This pathway has been poorly studied, but apparently Claudin (Cldn)-2 and Cldn-12 would be responsible for transporting Ca<sup>2+</sup> in the intestine<sup>[82]</sup>. Ca<sup>2+</sup> movement through the TJ is a non-saturable process, which depends on the concentration and the electric gradient across the epithelium. High Ca<sup>2+</sup> intakes switch on the paracellular route due to a short sojourn time in the intestine and a down-regulation of proteins involved in the transcellular pathway<sup>[83]</sup>. It has been observed that the expression of paracellular TJ genes is regulated by the transcellular calbindin protein, suggesting that active and passive Ca<sup>2+</sup> transport pathways may work cooperatively<sup>[84]</sup>.

## ACTIONS OF PRO-OXIDANTS ON INTESTINAL CALCIUM ABSORPTION

Twenty years ago, we reported that DL-buthionine-S,R-sulfoximine (BSO), an inhibitor of GSH biosynthesis, decreased the intestinal Ca<sup>2+</sup> absorption in vitamin D-deficient chicks treated with cholecalciferol. This response was reversed by addition of GSH monoester to the intestinal sac, demonstrating for the first time that the intestinal GSH normal levels are essential for an adequate intestinal Ca<sup>2+</sup> absorption. In vitamin D-deficient chicks without treatment, BSO did not affect the Ca<sup>2+</sup> transport and the GSH content beyond the low values already triggered by the vitamin deficiency<sup>[85]</sup>. The activity of intestinal alkaline phosphatase (IAP), an enzyme presumably involved in the intestinal Ca<sup>2+</sup> absorption, was also highly reduced by BSO in vitamin D-deficient chicks treated with vitamin D<sub>3</sub>. The effect of BSO was observed either *in vivo* or *in vitro*. BSO did not act directly on IAP, but it caused GSH depletion which led to changes in the redox state of the enterocyte, as evidenced by the  $\cdot$ HO production and an incremental increase in the protein carbonyl content. Again the reversibility of the BSO effect was demonstrated by addition of GSH monoester to the gut loop<sup>[86,87]</sup>.

Menadione (MEN) or vitamin K<sub>3</sub> is another pro-

oxidant compound that alters the intestinal Ca<sup>2+</sup> absorption *via* GSH depletion<sup>[88]</sup>. MEN metabolism involves redox cycling, resulting in the release of ROS. MEN may undergo one or two-electron reduction. When MEN suffers one-electron reduction, there is formation of very unstable semiquinone radicals; they react rapidly with O<sub>2</sub> resulting in regeneration of the parent compound and production of O<sub>2</sub><sup>-</sup> yielding H<sub>2</sub>O<sub>2</sub> through enzymatic or spontaneous dismutation. Two-electron reduction of MEN by DT-diaphorase produces hydroquinone, a pathway that constitutes a detoxification mechanism<sup>[89]</sup>. GSH is the electron donor in both cases, which explains the depletion of the intestinal tripeptide after MEN treatment. GSH depletion triggers oxidative stress as demonstrated by generation of  $\cdot$ HO and O<sub>2</sub><sup>-</sup> groups and an increase in the protein carbonyl content, which deteriorate the activities of enzymes or proteins involved in the Ca<sup>2+</sup> movement from lumen to blood. In fact, the activities of IAP and the plasma membrane Ca<sup>2+</sup>-ATPase as well as the expression of PMCA<sub>1b</sub>, calbindin D<sub>28k</sub> and Cldn-2 were decreased by MEN treatment<sup>[88,90]</sup>. At the studied doses, the inhibitory action of MEN on intestinal Ca<sup>2+</sup> absorption began in half an hour, lasted for several hours and finished after 9 or 10 h of treatment, indicating that the effect was transient, probably because the intestine could reinforce its ability to overcome the oxidative stress<sup>[88]</sup>. The inhibitory effect of MEN on intestinal Ca<sup>2+</sup> absorption implied intestinal mitochondrial dysfunction. As mentioned above, the optimal intestinal Ca<sup>2+</sup> absorption needs the integrity of intestinal mitochondria because it is the main source of metabolic energy. MEN caused mtGSH depletion, but it rapidly normalized. However, the mitochondrial membrane potential decreased and, simultaneously, cytochrome c was released from the intermembrane space to the cytoplasm, at least in mature enterocytes, which suggested triggering of apoptosis. In fact, this process was confirmed by DNA fragmentation that occurred in the 30%-40% of enterocytes, without affecting 60%-70% of the absorptive cells. In other words, the inhibitory effect of MEN on intestinal Ca<sup>2+</sup> absorption was partial and transient. The activity of two oxidoreductases from the Krebs cycle, malate dehydrogenase and  $\alpha$ -ketoglutarate dehydrogenase, was reduced by MEN in 18% and 30%, respectively. This means that the majority of mitochondria remained competent for ATP synthesis, making possible the process of apoptosis<sup>[91]</sup> and a poor intestinal Ca<sup>2+</sup> absorption. MEN not only produced intestinal apoptosis through the mitochondrial pathway, but also by triggering the expression of FAS/FASL/caspase-3<sup>[92]</sup>. Although an enhancement in the Cu<sup>2+</sup>/Zn<sup>2+</sup>-SOD, CAT, GPx and Mn<sup>2+</sup>-SOD activities could represent cytoprotective mechanisms against the oxidant effects, they were insufficient to avoid an inhibition in the overall process of intestinal Ca<sup>2+</sup> absorption<sup>[92-94]</sup>. The results supported previous data showing alterations

in the intracellular thiols and Ca<sup>2+</sup> homeostasis, ATP depletion and DNA breakage after toxic MEN concentrations<sup>[95-97]</sup>.

Sodium deoxycholate (NaDOC), a sodium salt of a major hydrophobic bile acid, also inhibits the intestinal Ca<sup>2+</sup> absorption at high physiological doses. This inhibition is time and dose dependent. We have demonstrated that PMCA<sub>1b</sub> decreased by the bile salt and the same occurred with the protein expression of PMCA<sub>1b</sub>, calbindin D<sub>28k</sub> and NCX1. NaDOC also produced GSH depletion, as well as ROS generation and mitochondrial swelling, which in turn led to mitochondria mediated apoptosis. Briefly, a single high concentration of NaDOC inhibits intestinal Ca<sup>2+</sup> absorption *via* downregulation of proteins involved in the transcellular pathway, as a result of oxidative stress and apoptosis<sup>[98]</sup>. Similarly, in a rat model of type 1 Diabetes mellitus induced by streptozotocin, we have also demonstrated oxidative stress in the intestine at early stages of developing of disease, leading to inhibition of the intestinal Ca<sup>2+</sup> absorption. Simultaneously, time-dependent adaptive mechanisms triggered an increment in the protein expression of molecules involved in both the transcellular and the paracellular pathways, which contributes to normalizing the intestinal Ca<sup>2+</sup> absorption as well as the duodenal redox state<sup>[99]</sup>.

Diets rich in fat also produce redox imbalance and increased oxidative stress in the duodenum causing down-regulation of calbindin D<sub>9k</sub>, PMCA<sub>1b</sub> and NCX, and consequently, an inhibitory effect on intestinal Ca<sup>2+</sup> absorption<sup>[100]</sup>. Orihuela *et al.*<sup>[101]</sup> have found that aluminium interferes with Ca<sup>2+</sup> uptake by enterocytes through a decrease in the intestinal GSH level affecting calbindin D function and/or synthesis, which leads to a reduced transcellular Ca<sup>2+</sup> absorption. Wu *et al.*<sup>[102]</sup> have reported that advanced oxidation protein products decrease the expression of Ca<sup>2+</sup> transporters in small intestine *via* the p44/42 MAPK signaling pathway. They consider that these data are relevant to understanding the mechanisms of IBD-associated osteoporosis.

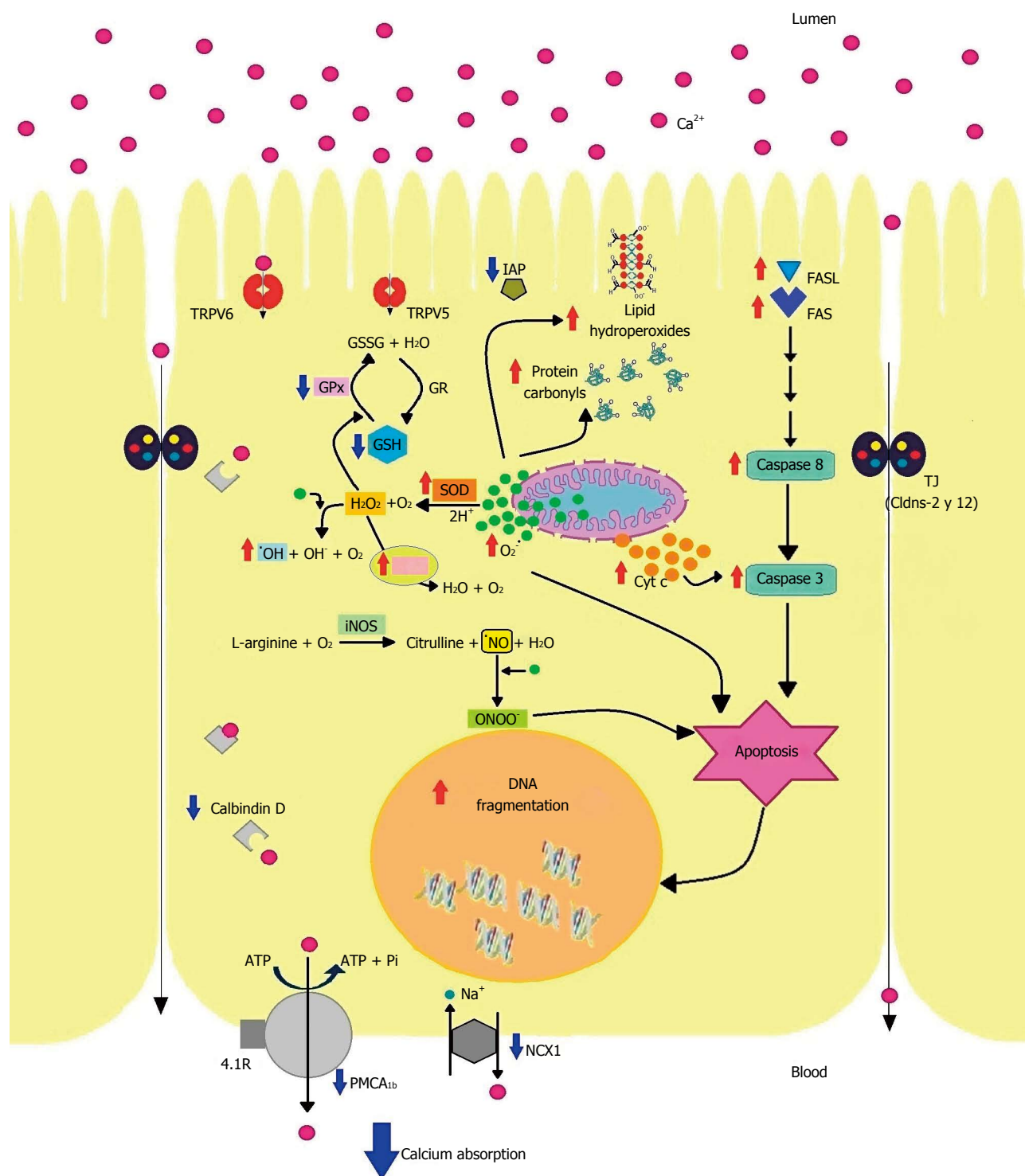
In summary, not only drugs but diet components or pathophysiological conditions that occur with GSH depletion or increased oxidative stress are deleterious for the intestinal Ca<sup>2+</sup> absorption because they alter the protein expression and/or activities of molecules involved in the transcellular and/or paracellular Ca<sup>2+</sup> pathways. Figure 1 is a schematic representation of the possible mechanisms involved in the inhibition of intestinal Ca<sup>2+</sup> absorption caused by oxidative stress.

## ANTIOXIDANTS AND THEIR MOLECULAR MECHANISMS FOR THE PRESERVATION OF INTESTINAL CALCIUM ABSORPTION

As mentioned earlier, endogenous enzymatic and nonenzymatic compounds defend the cells under

oxidant conditions<sup>[40]</sup>. However, when there is a noticeable shift to the oxidation, they cannot respond adequately. It has been suggested that natural or synthetic compounds would help to overcome the disequilibrium<sup>[103,104]</sup>. In our laboratory, we have demonstrated that the inhibition of intestinal Ca<sup>2+</sup> absorption caused by oxidants, mainly causing GSH depletion, could be either prevented or restored by quercetin<sup>[92]</sup> (QT, a plant derived flavonoid), melatonin<sup>[90,94]</sup> (MEL, a natural antioxidant present in humans), lithocholic (LCA)<sup>[105]</sup> or ursodeoxycholic (UDCA)<sup>[106]</sup> acids (bile acids less hydrophobic than deoxycholic acid). QT is a polyphenolic flavonoid found in several fruits and vegetables of the human diet<sup>[107]</sup>, mainly highly concentrated in onions, tea and apples<sup>[108]</sup>. It is a potent scavenger of ROS with various pharmacological properties such as anticancer-activity, anti-virus and anti-inflammatory effects reducing the risk of cardiovascular and renal diseases<sup>[109,110]</sup>. QT inhibits enzyme systems responsible for the generation of ROS (cyclooxygenase, lipoxygenase and xanthine oxidase)<sup>[111]</sup>, binds to superoxide anions, singlet oxygen and hydroxyl radicals, and as a consequence reduces lipid peroxidation<sup>[112]</sup>, chelates transition metals such as iron and copper<sup>[113,114]</sup>, and inhibits the aldose reductase activity<sup>[115]</sup>. We could demonstrate that QT protects the intestinal Ca<sup>2+</sup> absorption against the inhibition provoked by MEN, but by itself does not affect it. Similarly, QT abolishes the GSH depletion caused by the quinone, but QT alone does not modify the intestinal GSH content. The flavonoid also avoids changes in the mitochondrial membrane permeability and abrogates the activation of FASL/FAS/caspase-3 pathway caused by MEN<sup>[92]</sup>. Conclusively, QT may be useful to prevent the inhibition of intestinal Ca<sup>2+</sup> absorption caused by MEN or other GSH depleting drugs by blocking the oxidative stress and apoptosis. In contrast, the soy isoflavones have shown a lack of dose-responsive on transepithelial Ca<sup>2+</sup> transport in human intestinal-like Caco-2 cells<sup>[116]</sup>, although they may reduce bone loss in postmenopausal women, which suggests that they act directly on bone cells.

We have also demonstrated that MEL may also restore the intestinal Ca<sup>2+</sup> absorption altered by MEN<sup>[94]</sup>. MEL is an indolamine that is present in all phyla of multicellular animals<sup>[117]</sup>. Although its main site of synthesis is the pineal gland, MEL is synthesized in other extracellular sites such as the intestine<sup>[118]</sup>, where the MEL level is 400 times larger than that from the pineal gland<sup>[119]</sup>. It has been shown that MEL scavenges ROS and protects against the deleterious effects of I/R through a stimulation of certain antioxidant enzymes preserving cellular energy and preventing mitochondrial damage<sup>[120,121]</sup>. In our study, we have shown that MEL blocks the inhibition of the intestinal Ca<sup>2+</sup> absorption caused by MEN, at least in part, by increasing the activity of antioxidant enzymes, returning GSH and protein carbonyl values to control levels, and rescuing the epithelial cells from



**Figure 1** Schematic representation of the possible mechanisms involved in the inhibition of intestinal  $\text{Ca}^{2+}$  absorption caused by oxidative stress. TRPV6: Transient receptor potential vanilloid 6; TRPV5: Transient receptor potential vanilloid 5; IAP: Intestinal alkaline phosphatase; GSSG: Disulfide of glutathione; GSH: Glutathione; GPx: Glutathione peroxidase; GR: Glutathione reductase; SOD: Superoxide dismutase; CAT: Catalase; Cyt c: Cytochrome c; Cldns: Claudins; iNOS: Inducible nitric oxide synthase; NCX1: Intestinal  $\text{Na}^+/\text{Ca}^{2+}$  exchanger; PMCA1b: Plasma membrane  $\text{Ca}^{2+}$ -ATPase 1b; TJ: Tight junctions.

apoptosis<sup>[94]</sup>. More recently, we have also proven that MEL not only restores but also prevents the inhibition of intestinal  $\text{Ca}^{2+}$  absorption provoked by GSH depleting drugs such as MEN and BSO<sup>[90]</sup>. MEL restores partially both the transcellular and paracellular  $\text{Ca}^{2+}$  pathways altered by the quinone, through dampening the  $\text{O}_2^{\cdot -}$  levels without affecting the  $\cdot\text{NO}$  system. MEL

was able to return the decreased protein expression of calbindin  $\text{D}_{28\text{K}}$  and Cldn-2 caused by MEN to the control values, but it could not restore the levels of PMCA1b. As MEL has beneficial effects on both  $\text{Ca}^{2+}$  transport mechanisms, it might improve the intestinal  $\text{Ca}^{2+}$  absorption under conditions of low or adequate Ca intake. The modulation of  $\text{Ca}^{2+}$  transporters by MEL

has also been reported in pancreatic acinar cells<sup>[122]</sup> and in pituitary cells<sup>[123]</sup>. Another mechanism by which MEL blocked the inhibition of intestinal  $\text{Ca}^{2+}$  absorption was the attenuation of the mitochondrial dysfunction in the duodenum, which has been also observed to be produced by MEL in other tissues and cells<sup>[124-126]</sup>.

Two other molecules with antioxidant properties such as UDCA and LCA were shown to block the inhibitory effect of NaDOC on intestinal  $\text{Ca}^{2+}$  absorption. UDCA and LCA are two bile acids with different solubility, chemical properties and physiological functions<sup>[127]</sup>. UDCA is a non-toxic hydrophilic bile acid used for treatment of gallstones and primary biliary cirrhosis (PBC)<sup>[128]</sup>. UDCA is naturally present in humans in a concentration of about 1%-3% of the total bile acid pool. When used in PBC treatment, its concentration increases to 40%-60%, making UDCA the predominant bile acid. The hydrophilicity of bile *via* UDCA serves to ameliorate cholestasis and minimize toxicity<sup>[129]</sup>. At the intestinal level, we have shown that UDCA increases the  $\text{Ca}^{2+}$  absorption modulating positively the  $\text{Ca}^{2+}$  uptake by mature enterocytes, which occurs in part as a result of increasing the vitamin D receptor (VDR) unit numbers<sup>[106,130]</sup>. When UDCA is simultaneously administered with NaDOC, UDCA avoids the inhibitory effect of NaDOC on intestinal  $\text{Ca}^{2+}$  absorption. One of the molecular mechanisms involved in this response is the attenuation of the apoptotic extrinsic pathway triggered by NaDOC. UDCA by itself decreases FAS and FASL protein expression and neither alters caspase 8 protein expression nor caspase 3 activity. In the presence of NaDOC, UDCA avoids the apoptotic effect of NaDOC normalizing the protein expression of FAS, FASL, caspase-8 and the enzyme activity of caspase-3. The NaDOC induced apoptosis is mediated by increment in the NO content and in the iNOS protein expression, effects that were abolished by UDCA. Another molecular mechanism triggered by UDCA is to avoid the enhancement in the LC3 II protein expression and the number of acidic vesicular organelles in the presence of NaDOC. In other words, UDCA avoids efficiently not only NO induced apoptosis, but also autophagy triggered by NaDOC<sup>[130]</sup>.

LCA is a secondary bile acid produced by the intestinal microflora. It binds to VDR<sup>[131]</sup>, has antibacterial activity<sup>[132]</sup>, produces antiproliferative and pro-apoptotic effect on human cancer cell lines<sup>[133,134]</sup>, inhibits proteasome<sup>[135]</sup>, acts as a membrane pore<sup>[136]</sup> and has anti-aging properties<sup>[137]</sup>. It is worldwide recognized that  $1,25(\text{OH})_2\text{D}_3$  is the main stimulator of the intestinal  $\text{Ca}^{2+}$  absorption, and both LCA and  $1,25(\text{OH})_2\text{D}_3$  are VDR ligands, although they have different VDR binding affinity<sup>[138]</sup>. In a recent study, we have demonstrated that neither the intestinal  $\text{Ca}^{2+}$  absorption nor the redox state of enterocytes is changed by LCA alone. Interestingly, LCA did not alter the intestinal  $\text{Ca}^{2+}$  absorption but prevented the inhibitory effect of NaDOC<sup>[105]</sup>. LCA blocked a decrease caused by NaDOC

on gene and protein expression of molecules involved in the transcellular pathway of intestinal  $\text{Ca}^{2+}$  absorption, ameliorated changes in mitochondrial membrane permeability and abrogated the enhancement in the protein expression of molecules from the apoptotic extrinsic pathway<sup>[105]</sup>. In addition, the simultaneous treatment of NaDOC and LCA blocked the oxidative stress caused by NaDOC, which indicates that LCA shows antioxidant and antiapoptotic properties in the presence of a pro-oxidant molecule as NaDOC. The functional toxicity of LCA in humans is in question due to the efficient human detoxification<sup>[139]</sup>, therefore, the use of LCA to protect the intestinal  $\text{Ca}^{2+}$  absorption under oxidant conditions caused by medications or pathological conditions might become a possible therapeutic strategy.

## CONCLUSION

The optimal intestinal  $\text{Ca}^{2+}$  absorption is highly dependent on the intactness of intestinal GSH content. GSH depleting drugs such as BSO, MEN or NaDOC trigger oxidative stress, leading to apoptosis and/or autophagy to finally produce inhibition of intestinal  $\text{Ca}^{2+}$  absorption. Similarly, pathological conditions associated with intestinal GSH depletion provoke oxidative stress and, hence, inhibition of intestinal  $\text{Ca}^{2+}$  absorption, as occurs in type 1 diabetes mellitus. The use of antioxidants could be a therapeutic strategy to protect or to restore the intestinal normal redox state maintaining an adequate intestinal  $\text{Ca}^{2+}$  absorption. QT, MEL, UDCA and LCA have been proven to be successful to normalize the  $\text{Ca}^{2+}$  transfer from lumen-to-blood in experimental animals under oxidant conditions. Therefore, they could be drugs of choice for the treatment of altered intestinal  $\text{Ca}^{2+}$  absorption in pathophysiological conditions such as diabetes, celiac disease, IBD, aging and other disorders associated with intestinal oxidative stress.

## ACKNOWLEDGMENTS

Prof. Dr. Nori Tolosa de Talamoni is a Member of Investigator Career from Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET). Solange Guizzardi and Luciana Moine are recipients of Doctoral Fellowships from CONICET.

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**P- Reviewer:** Lasekan J, Prasad S **S- Editor:** Yu J **L- Editor:** A  
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