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Cytoprotective mechanisms in rats lung parenchyma with zinc deprivation

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Abstract Suboptimal intake of Zinc (Zn) is one of the most common worldwide nutritional problems. The aim of this study is to provide new evidence on the relation between moderate Zn restriction, and cytoprotective functions in airway epithelium. We analyzed the effect of moderate Zn deficiency (ZD) on the expression of several pro and anti-apoptotic proteins and cytoprotective factors (Hsp27 and Hsp 70i), as well as the effect of restoring Zn during the refeeding period. Adult male rats were divided into three groups: Zn-adequate control group, Zn-deficient group and Zn-refed group. Our previous findings showed an important oxidative and nitrosative stress during ZD, this situation is accompanied by inflammation and alterations in the expression of matrix extracellular proteins. We observed a strong immunopositive area of anti and pro-apoptotics proteins in ZD groups. The mRNA levels of Nrf-2, Bax and Bad were increased in ZD, while in ZD refed group its levels were similar to the control values. The increased expression of Nrf-2 is likely to be critical for protection of lung under inflammatory process triggered during ZD. Hsp27 and Hsp 70i showed an increase of immunostaining area but they were not significant. During the supplementation period, heat-shock proteins increased significantly. In conclusion, our results provide further evidence of the pathways involved in cytoprotection and apoptosis caused by ZD. Additional studies are required in order to investigate whether Hsp27 and Hsp70 are consistently associated with cellular stress and inflammation in lung. There may be a beneficial role for improved Zn nutrition or Zn supplements early in lung pathology.

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Introduction

Zinc (Zn) deficiency is estimated not only to be present in developing countries but also to occur frequently throughout the world as mild to moderate deficiency. This deficiency may contribute to the severity of infectious diseases and mortality in malnourished children and Zn supplementation has been shown to reduce the severity of diarrhea and the incidence of infections. Zn has particular importance in maintaining homeostasis of epithelial tissues that are at the front line of defense, such as pulmonary alveoli (Zhu et al. 2007). Bao and Knoell (2006a) reported that Zn acts as a cytoprotector of lung epithelium during inflammatory stress and that cellular depletion of Zn enhances susceptibility to apoptosis.

During the apoptotic process, the molecular targets for Zn are the anti-apoptotic Bcl-2-like and pro-apoptotic Bax-like mitochondrial membrane proteins, among others. The expression of Bax and Bcl-2 protein is upregulated in alveolar epithelial cells and the number of apoptotic epithelial cells is associated to the prognosis of patients with diffuse alveolar damage (Kazuyoshi 2007).

Zn deficiency (ZD) may increase oxidative stress (Song et al. 2009). We previously showed that ZD state increased the oxidative and nitrosative stress in lung parenchyma, which release several pro inflammatory factors and provoke an infiltration of inflammatory cells, such as neutrophils (Biaggio et al. 2010; Gomez et al. 2006). There are also some evidence indicating that oxidative stress could modulate the activity of transcriptors factors as Clusterine/Apolipoprotein J (CLU) or nuclear factor erythroid 2-related factor 2 (Nrf2). CLU is an extremely sensitive biosensor of environmental changes of reactive oxygen species (ROS) and it has been implicated in processes such as, lipids transport, cell differentiation, cell–cell and cell–matrix interactions, as well as in the regulation of the apoptotic pathway by either promoting or suppressing cell death (Balantinou et al. 2009). On the other hand, recent studies have demonstrated a clear link between defects in the lung antioxidant defense system, regulated by nuclear factor erythroid 2-related factor 2 (Nrf2) and excessive oxidative stress, increased apoptosis, inflammation and exacerbated emphysema (Bao and Knoell 2006b). In order to dilucidate if any of this transcription factors were involved in the response developed in ZD we decided to measure the levels of mRNA of both factors.

Heat-shock proteins (Hsps) are a group of molecular chaperone proteins that were shown to be induced by a variety of stresses. Recently, antioxidant enzymes and Hsps have been broadly used as molecular biomarkers to reveal the potential cellular and physiological effects of exogenous factors, such as microorganism, chemicals, deleterious materials in diet, and malnutrition (Wu et al. 2011; Verlecar et al. 2007). Therefore, expression of Hsps may be used as a sensitive biomarker when cells are placed under conditions of stress (Han et al. 2007). Nevertheless although Hsps are cytoprotective, cells exposed to extreme or prolonged stresses undergo cell death by necrosis or apoptosis despite the expression of higher concentrations of Hsps. Hsp27 is a small molecular chaperone with an ability to interact with a large number of proteins. Recent evidence has shown that Hsp27 regulates apoptosis through an ability to interact with key components of the apoptotic signalling pathway, in particular, cytochrome C and procaspase-3 (Concannon et al. 2001). The overexpression of Hsp27 can confer resistance to apoptosis during the differentiation of monocytes to macrophages (Han et al. 2007).

Considering our previous findings, the aim of this study is to provide new evidence on the relation between moderate Zn restriction, and cytoprotective functions in airway epithelium. We studied the expression of Hsp27 and Hsp70i. We also investigated whether changes in cytoprotective functions are associated to alterations in Nrf-2 or CLU expression and in the apoptotic cascade. Lastly, we studied whether the supplementation with Zn would recover the different parameters to its normal/control values/levels.

Materials and methods

Diet and experimental design

Wistar adult male rats (200 ± 10 g) were fed two diets with different Zn concentration, during 8 weeks. Pretest, rats with similar intake profiles were matched and randomly assigned to the control or the Zn

deficient groups, each group contain fourteen animals. For that purpose, rats were separated into three groups and fed respectively:

1. Zn-deficient diet (ZD) containing 5 mg Zn/kg (as ZnCl2).
2. Zn adequate control diet (CO) containing 30 mg Zn/kg (as $ZnCl_2$).
3. A group of deficient animals was fed with the control diet 10 days before sacrifice (ZD refed group) in order to supply these animals with ion Zn.

All the other components of the diet remained constant, and were supplemented with recommended amounts of vitamins and minerals, according to AIN 93-M (America Society for Nutritional Sciences 93-M) diet (Reeves et al. 1993). Both diets had the following composition (g/kg): 466 cornstarch, 140 casein (785 % protein), 155 dextrinized cornstarch, 100 sucrose, 50 fiber/cellulose, 40 soybean oil (containing liposoluble vitamins), 35 mineral mix AIN-93M-MX (Zn was not incorporated into the mineral mix of ZD diet), 10 vitamin mix (AIN-93-VX), 1.8 L-cystine, 0.008 ascorbic acid, 2.5 choline bitartrate (41 % choline).

All dietary ingredients were monitored for Zn concentration using atomic absorption spectrophotometry (AAS). Animals were housed individually in a controlled environment with a 12 h light: 12 h darkness cycles, temperature was maintained at 21 ± 2 °C. Fresh diets were given and left over food discarded on a daily basis. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) (US Public Health Service 1985) and the National University of San Luis Committee's Guidelines for the Care and Use of Experimental Animals (ordinance CD 006/02). Diet acclimation lasted 1 week, during that time, control diet was provided to all rats and intake was measured.

Serum and tissue collection

At the second month of treatment and 12 h after the last feeding, the animals were killed. Previously, they were anesthetized intraperitoneally (IP) with sodium pentobarbital (50 mg/kg), and blood samples for determination of Zn serum were collected. The lungs were quickly removed, washed with ice-cold 0.9 % saline solution and weighed. Serum and pieces of lobes of each lung were frozen in liquid nitrogen, and then they were kept at −80 °C until they were analyzed. For immunochemistry the tissues samples were fixed in 10 % buffered formalin during 6 h at room temperature and washed in phosphate-buffered saline (PBS). For light microscopy the fixed tissues were dehydrated, cleared in xylene, and embedded in paraffin. Five-micrometer thick sections were mounted in slides previously treated with 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO).

Zn analysis

Aliquots of the diet, serum and lung were collected without allowing any contact with metal. Each sample was wet-ashed with 16 N nitric acid as described by Clegg et al. (1981). Zn concentrations of the pretreated samples and serum were quantified by an atomic absorption spectrophotometer (model 5100, HGA-600 Graphite Furnace, Perkin-Elmer, USA). A linear calibration curve using certified standard NIST (National Institute and Standard and Technology) solutions was carried out. All specimens were diluted by bidestilled, deionized water, and analyzed by duplicate. Before sample digestion, different amounts of standard solution of each element were added. Recovery was between 98 and 99.2 % for different elements.

Immunohistochemistry

The primary antibodies used were caspase-3 active (Clone: polyclonal, R&D systems (USA), without antigen retrieval; dilution 1:500); Bcl-2 (Clone: Bcl-2, polyclonal antibody, Zymed San Francisco CA (USA); dilution: 1:200); Bax (EG3) (Clone Bax; polyclonal antibody; Zymed San Francisco CA (USA); dilution 1:250) Bcl-L2 (Clone: Bcl-w; polyclonal antibody, Zymed San Francisco CA (USA), dilution: 1:500); Hsp 25/27 (anti-rabbit against hybrid Hsp 25/27, this antibody was kindly provided by Dr. M. Gaestel (Max-Delbrück Center for Molecular Medicine, Berlin, Germany) and Hsp70/Hsp72, mAb (C92F3A-5) (Clone: Hsp 70 inducible, monoclonal antibody; Stressgen, dilution 1:1,000). The secondary antibodies we used were Anti-rabbit IgG (polyclonal, Zymed San Francisco CA (USA), dilution 1:300) and Anti-rabbit IgG (polyclonal, Chemicon, dilution 1:500). A streptavidin-: biotin immunoperoxidase method was done as previously described (Salvetti

et al. 2004; Ortega et al. 2004). The endogen peroxidase activity was inhibited with 1 % (v/v) H_2O_2, and non-specific binding was blocked with 10 % (v/v) normal goat serum. All sections were incubated with primary antibodies for 18 h at 4 °C and then for 30 min at room temperature with rat-preabsorbed biotinylated secondary 1:5 or polyclonal). The visualization of antigens was achieved by the streptavidin–peroxidase method (BioGenex, San Ramon, CA, USA), and 3.3-diaminobenzidine (DAB; Dako, Carpinteria, CA, USA) was used as chromogen. Finally, the slides were washed in distilled water, counterstained with Mayer's hematoxylin, dehydrated and mounted.

Image analysis

Image analysis was performed using an Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MA, USA). For the immunohistochemistry technique, images were digitized by a CCD colour video camera (Sony, Montvale, NJ, USA) mounted on a conventional light microscope (Olympus BH-2, Olympus Co., Japan), using an objective magnification of 40×. This was achieved by recording a reference image of an empty field for the correction of unequal illumination (shading correction) and by calibrating the measurement system with a reference slide to determine background threshold values. The reference slides contained a series of tissue sections stained in the absence of a primary antibody. The methodological details of image analysis have been described earlier (Ranefall et al. 1998; Ortega et al. 2007; Salvetti et al. 2007; Wang et al. 1999, 2000). The average density (% of immunopositive area) was calculated as a percentage of total area evaluated through color segmentation analysis, which extracts objects by locating all objects of a specific color (brown stain). These values were verified and normalized with the controls carried across various runs using the same region (verified by image comparison) for calibration. Sections were analyzed with the observer blinded to treatment. The images were then transformed to a bi-level scale TIFF (tagged image file format) format.

RNA isolation and RT-PCR analysis

A lobe of the lung was used for total RNA analyses. Total RNA was isolated by using TRIzol (Life Technologies). RNA isolations were performed as instructed by the manufacturers. The purity and integrity of the samples were confirmed by electrophoretic assay with ethidium bromide staining. Quantification of RNA was based on spectrophotometric analysis at 260/280 nm. 10 μg of total RNA were reverse-transcribed with 200 units of MMLV Reverse Transcriptase (Promega Inc.) using random hexamers as primers in a 20 μl reaction mixture, following the manufacturer's instructions. PCR was performed in 35 μl of reaction solution containing 0.2 mM, dNTPs, 1.5 mM $MgCl_2$, 1.25 U of Taq polymerase, 50 pmol of each rat specific oligonucleotide primers and RT products (1/10 of RT reaction). The samples were heated to 94 °C for 2 min, followed by 38 temperature cycles. Each cycle consisted of three periods: (1) denaturation, 94 °C for 1 min; (2) annealing, 58 °C for Bax and CLU; 55 °C for Bad and β-actin; Nrf-2 59 °C; during 1 min; (3) extension, 72 °C for 1 min. After 38 reaction cycles, the extension reaction continued for 5 more minutes. The PCR products were electrophoresed on 2 % agarose gel with 0.01 % ethidium bromide. The image was visualized and photographed under UV transillumination. The PCR reaction was carried out using a forward primer specific to rat Bad 5′ GAAGGGATGGGGGAGGAGC 3′and reverse primer 5′ CGATCCCACCAGGACTGGA 3′, a forward primer specific to rat Bax 5′ ATGGACGGGTCCGGGG AGC 3′ and reverse primer 5′ TCAGCCCATCTTCT TCCAGAT 3′, a forward primer specific to rat CLU 5′ TACAGTTCCCGATGTGGAT 3′ and reverse primer 5′ CACGAGAGGGGACTTCTGAG 3′, a forward primer specific to rat Nrf2 5′CGCCATTTCACTGAACACAA GT 3′ and reverse primer 5′TGGCTGCTTTAGGTCC-ATT 3, a forward primer specific to rat β-actin 5′CGTG GGCCGCCCAGGCACCA 3′ and reverse primer 5′TTGGCCTTAGGGTTCAGAGGG3′. RT-generated fragments code for Bcl-2 associated X protein Bax (Metcalfe et al. 1999); Bad (Metcalfe et al. 1999); CLU (Rosenberg and Silkensen 1995); Nrf-2 (Thimmulappa et al. 2002) and β-actin (Choi and Choi 2000). The expected PCR products were for Bad 270 bp, Bax 365 bp, CLU 402 bp, Nrf-2 160 pb and β-actin 243 bp. The intensity of each band was measured using the NIH Image software and reported as the values of band intensity units. The relative abundance of each target band was then normalized according to the housekeeping gene β-actin, calculated as the ratio of the intensity values of each target product to that of β-actin. β-Actin was chosen as the reference protein, over glyceraldehyde 3-phosphate dehydrogenase (GAPDH), for the better linearity of expression (data not shown).

Table 1 Body and lung weight and zinc concentrations in serum and lung of male rat

	CO (control)	ZD (deficient)	ZDrefed
Body weight (g)	396 ± 24	338 ± 33*	407 ± 56+
Zn in lung (ppm)	3.2 ± 0.5	1.4 ± 0.2*	2.2 ± 0.4
Zn in serum (μmol/l)	6.7 ± 1.2	4.1 ± 0.5*	7.2 ± 1,0++

Results given as mean ± SEM, n = 14 for each case. Across a row, values with different superscript indicate differences by ANOVA test (*) $p < 0$, 05 when compared Co versus ZD; (+) $p < 0$, 05; (++) $p < 0$, 01 when we compared ZD versus ZDrefed. *CO* control group, *ZD* zinc deficient group, *ZDrefed* zinc supplementation group, *ppm* parts per million

Statistical analysis

All data are expressed as mean ± SEM. For comparisons that involved three experimental groups, one way ANOVA (GraphPad Prism 5.0, software) were used (Denenberg 1984). Having passed statistical significance by ANOVA, individual comparisons were made with the Dunnett's multiple-comparison test. Differences between means were considered significant at the $p < 0.05$ level.

Results

Weight gain and Zn status of the rats

After 2 months of treatment, the ZD group body weight was significantly lower than the control; while the ZD refed group was significantly higher when compared to ZD group. In serum and tissue, Zn concentrations were significantly decreased in ZD group, and in ZD refed group the level of Zn overtook the control values. After treatment, clinical signs of ZD were; lower gloss coat, alopecia and aggressiveness, but other signs such as dermatitis was not observed (Table 1).

Alterations in Nrf2, Bad, Clusterine and Bax genes expression during Zn Deficiency (RT-PCR)

In our experimental model, we observed that the expression of nuclear factor Nrf-2 was increased in the ZD group. This factor is strongly linked to the activation of several antioxidant enzymes, anti- and pro-apoptotic proteins and cytoprotective agents, such as Hsps. In Fig. 1, pro-apoptotic genes were increased in the ZD group, which indicates, perhaps, a response to the inflammatory process triggered during ZD. Our results showed a trend toward increased of CLU expression in both, ZD and ZD refed groups; however, it was not significant. It is known, that this protein has functions similar to Hsp, inhibiting activation of pro-apoptotic proteins and protecting cells from several stressors. There was no evidence of correlation between the expression of CLU and the increase of Nrf-2 and pro-apoptotic proteins.

Zn deficiency induces the activation of anti-apoptotic proteins

Having shown that Zn depletion enhances pro apoptotic proteins expression, we turned our attention on anti-apoptotic proteins Bcl-w protein expression increased two fold in ZD-group (**) $p < 0.01$, while Bcl-2 protein increased three fold in the same group, compared to the control.

Perhaps, in our experimental model, comparing pro- and anti-apoptotic status, the most striking results showed that the time of Zn supplementation or refeeding may be significantly reverse changes due to Zn deprivation (Fig. 2: Bcl-w: a–c; Bcl-2: d–f), Fig. 1.

Zn depletion induces caspase-3 activation and increase Bax expression

It is important to note, that several studies suggest roles of Hsps in different points of apoptotic cascade. In fact, Hsp 27 associates with caspase-3 pro-domain, inhibits the second proteolytic cleavage necessary for caspase-3 activation. Caspase-3 expression increased almost three fold in ZD group and four fold in ZD refed group, when compared to control. Other results showed an increase of immunostaining intensity of Bax in ZD group almost twice fold more than CO group. This situation is associated with increased expression previously showed by us, as expression of Bax (Fig. 3d–f).

Zn supplementation period protects lung parenchyma by activating heat shock proteins

Both, Hsp27 and Hsp 70i proteins were increased two-fold in ZD refed group, when compared to control

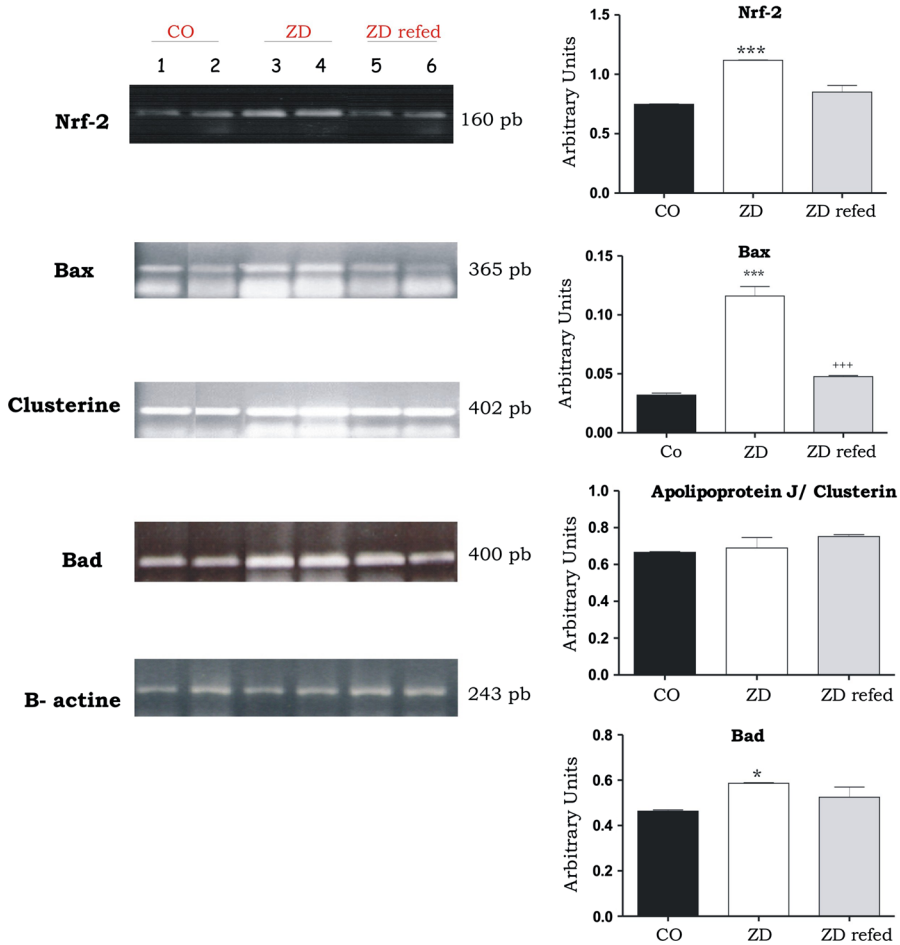

Fig. 1 Expression of Nrf-2 and pro-apoptotic proteins. Nrf-2 was increased in ZD vs CO group (***) $p < 0.001$. Both Bax and Bad proteins were increased in ZD vs CO group (*) $p < 0.05$ and (***) $p < 0.001$, respectively. During supplementation period, Bax expression was significantly decreased vs ZD group (^{+++}p) < 0.001. Apolipoprotein J/Clusterin did not change in all groups treated. β-actin was used as an internal control. *Lanes 1*, *2* control samples. *Lanes 3*, *4* ZD samples. *Lanes 5*, *6* ZDrefed samples. On the *right*, quantification of the intensity of the fragment bands in relation to the intensity of the internal control bands (n = 8 cases per group)

group. Again, our results showed that Zn supplementation period is enough to activate the expression of these proteins (Hsp27 and Hsp 70i) and stimulate cytoprotective and anti-apoptotic mechanisms in lung parenchyma (Fig. 4).

Discussion

Dietary ZD is globally common problem and it apparently contributes to the pathogenesis of diverse diseases. Besides dietary deficiency, low Zn levels are also present in patients with other abnormalities which could be compromising Zn absorption. This ion participates in cell replication, tissue repair, and growth and it is associated with an impaired immune system, an enhanced susceptibility to infections, as well as delayed wound healing (Murakami and Hirano 2008; Yanagisawa 2008). Moreover, this trace element acts as a cytoprotector during inflammatory stress and that cellular depletion enhances susceptibility to extrinsic apoptosis (Yousef et al. 2002).

In our experimental design, Zn deprivation for 2 months induces decrease of body weight in the ZD group and Zn refeeding restores the body weight in the ZD refed group (Table 1), according to previous results (Biaggio et al. 2010; Gomez et al. 2006; Ying et al. 2011). A decrease in serum Zn increased the risk for developing metabolic and clinical signs of ZD (Dorup and Clausen 1991) and confirmed previous results (Biaggio et al. 2010; Gomez et al. 2006). During the supplementation period, serum Zn concentration increased in relation to ZD. Some authors showed that when Zn is added back to the diet, the growth is promptly resumed, and proceeds even faster than in the controls (Dorup and Clausen 1991). In our work, the time of refeeding used was enough to overtake the normal level of Zn.

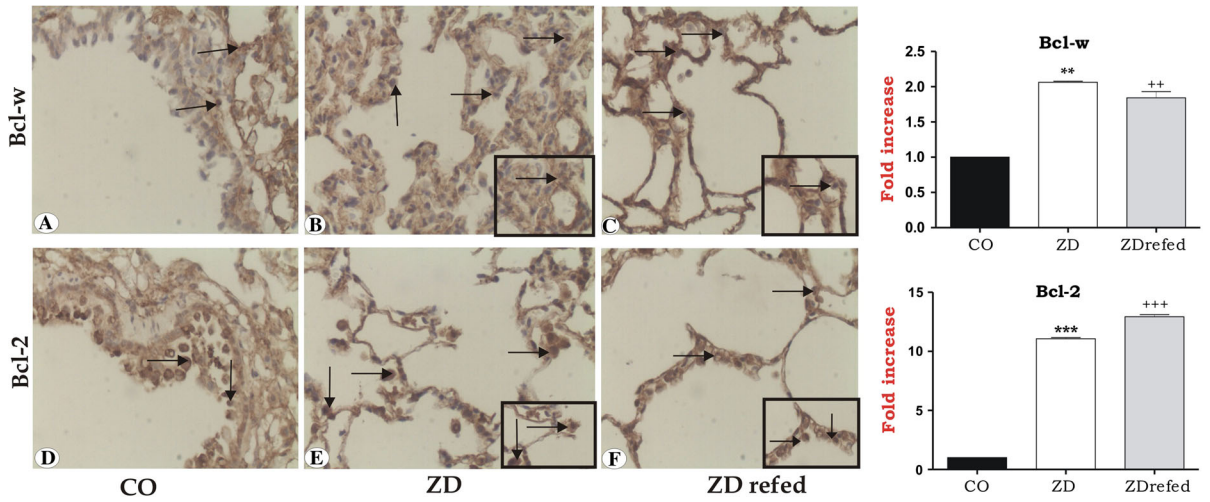

Fig. 2 Sections of rat lung parenchyma immunostained with Bcl-w and Bcl-2 antibodies. Both antibodies are present in alveolar cells. *Arrows* indicate the positive area for both antibodies used. Data in *graph* represent quantification of the intensity of the immunohistochemical expressed in fold. Inset in ZD and ZD refed groups reveals immunopositive area of each antibody. *Bcl-w* encoded by the Bcl-L2 gene (**a**–**c**) (**) and (++p) < 0.01 vs CO. *Bcl-2* (B cell lymphoma-2): anti-apoptotic protein (**d**–**f**) (***) and (+++p) < 0.001 vs CO. *CO* control group, *ZD* Zn deficient group, *ZD refed* Zn supplementation group. Magnifications ×40

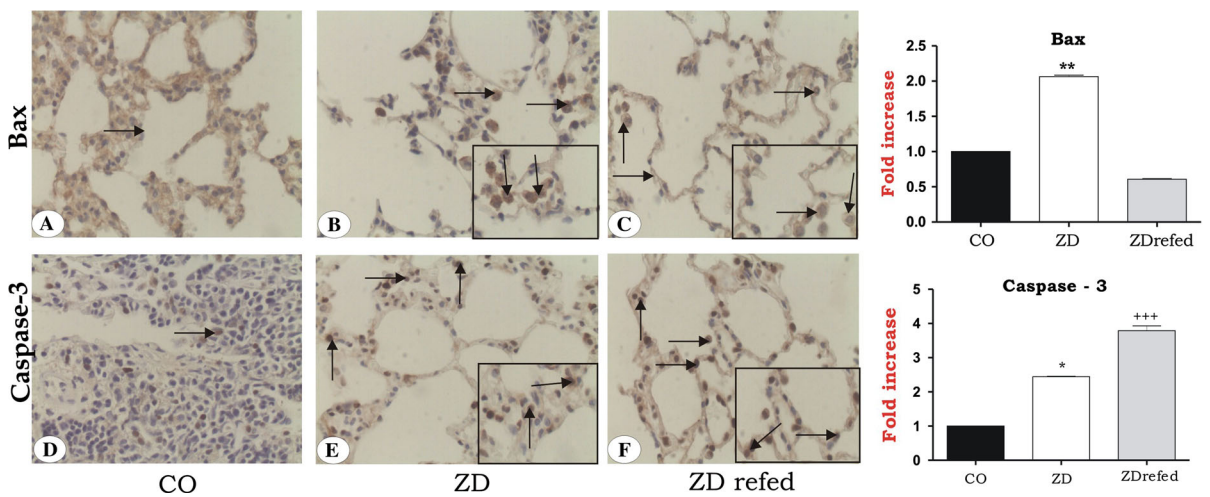

Fig. 3 Immunostaining of Bax and Caspase-3 in rat lung. Caspase-3 immunoreactivity appeared mainly in nuclear zone of cells. Bax positive immunostaining was detected in cytoplasmic region of cells. *Arrows* indicate the positive area for both antibodies used. *Bars* represent quantification of the intensity of the immunostaining expressed in fold. *Inset* in ZD and ZD refed groups reveals immunopositive area of each antibody. *Bax* (BaxEG3): pro-apoptotic protein (**a**–**c**) (**p) < 0.01 vs CO and *Caspase-3* cysteine-aspartic protease-3 (**d**–**f**) (*p) < 0.05 and (+++p) < 0.01 vs CO. *CO* control group, *ZD* Zn deficient group, *ZD refed* Zn supplementation group. Magnifications ×40

We analyzed Nrf-2 and CLU, because they are critical regulators of the response to oxidative stress in order to elucidate the pathway implicated in the production of antioxidant and cytoprotective gene response (Eom and Choi 2009, Blumenstein et al. 2013). Nrf-2 increases cell viability under different unfavorable conditions and it is involved in preventing apoptotic processes through different pathways (Wei et al. 2009; Reddy and Mossman 2002; Valko et al. 2006). The expression of Nrf-2 was increased in ZD when compared to control group, while in Zn supplemented animals the nuclear factor did not change its

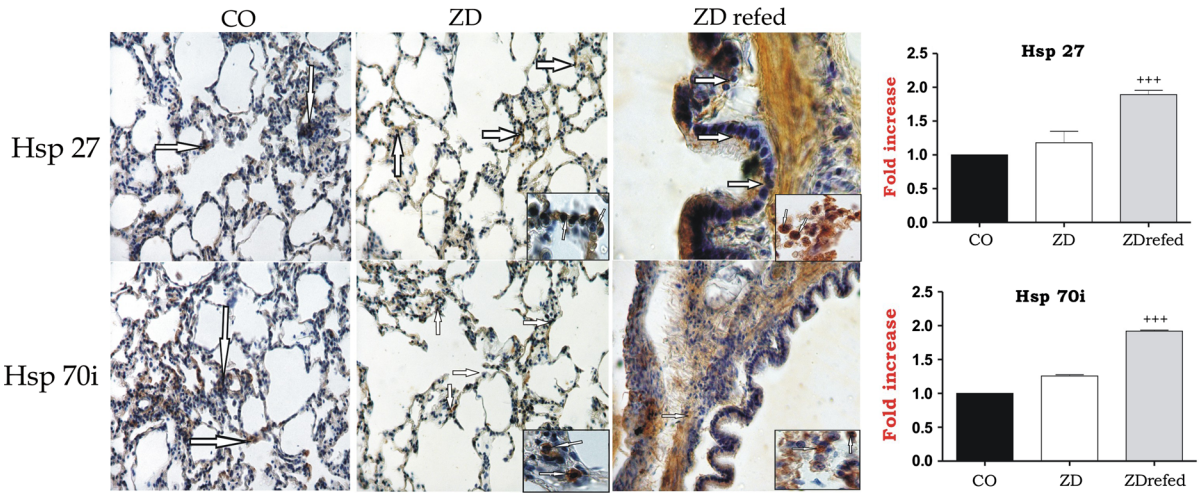

Fig. 4 Expression of Hsp-27 and Hsp-70i proteins in rat lung by immunochemistry. ZD and ZD refed groups showed an intense immunostaining in cytoplasm of alveolar cells. Hsp 27 and Hsp70i immunostaining showed a different profile of positive mark in nuclear zones (*inset* ZD and ZD refed groups). The intensity of positive immunostaining was quantified. ZD refed showed (+++p) < 0.01 vs CO in both studied proteins. *Arrows* indicate the positive area for both antibodies used. *CO* control group, *ZD* Zn deficient group, *ZD refed* Zn supplementation group. Magnifications $\times 40$

expression. We can assume that due to the stress caused by ZD. Nrf-2 might rise and increase cells survival in lung.

We have previously reported that nitric oxide related species (NO) are accumulated in lung during the course of ZD (Gomez et al. 2006). This is an interesting situation because NO and superoxide anion are produced at high levels, and can react extremely fast in order to form the potent oxidant peroxynitrite (Gomez et al. 2006). Overproduction of related nitric species results in "nitrosative" stress which contributes to a variety of pathological processes such as inflammatory diseases. This inflammatory condition can trigger apoptosis (Zalewski et al. 1993).

It is known that Zn has a protective role in preventing cell death by apoptosis, an event accompanied by the joint anti-apoptotic protein family Bcl-2 (Seve et al. 2002; Tapiero and Tew 2003). In our model of moderate ZD Bax showed an increased expression in ZD group, suggesting the activation of apoptosis in the lung, whereas during the supplementation period Bax mRNA levels decreased. Therefore, the period of 10 days was sufficient to reverse in part the situation and allowed some markers of apoptosis to reach the control values, reducing apoptosis by ZD. Bad and CLU expression did not change in the treated groups; however the expression of both proteins showed a trend to increase in ZD group.

The expression of the anti-apoptotic proteins Bcl-2 and Bcl-w were increased in the ZD group, what suggest the possible protection of lung parenchyma against the development of apoptosis. However, in ZD refed group the expression of Bcl-2 was greater than in the ZD group; suggesting that the lung parenchyma would revert the apoptotic process during the supplementation period.

Porcelli et al. (2008) and other authors found that Bcl-2 acts as an antioxidant to prevent damage to cellular components including membrane lipids (lipoperoxidation) and also its expression would be associated to glutathione levels increased (Porcelli et al. 2008; Korsmeyer et al. 1993). This evidence would suggest a possible relationship between oxidative stress and the expression of this anti-apoptotic protein. In previous studies we showed a significant increase in total glutathione so we can suggest, under these conditions, that the increased expression of Bcl-2 protein have antioxidant functions, as it was proposed (Porcelli et al. 2008; Gomez et al. 2003).

Moreover, it has also been observed that Zn and the protein Bcl-2 protect cells from oxidative stress (Truong-Tran et al. 2002) and suppress the activation of caspases (Thornberry and Lazebnik 1998). The expression of caspase-3 was increased in the ZD group, compared to the CO group, but in the ZD refed group caspase-3 expression did not decrease, when

compared to controls. This would indicate that the addition of Zn for 10 days would not be enough to counteract the apoptotic process. However, the mechanism by which ZD leads to activation of caspase-3 is not yet fully elucidated. Studies performed in epithelial cells ciliated trachea-bronchial, demonstrated the presence of high concentrations of Zn in the apical cytoplasm, in the same region where the precursor of caspase-3 is located (Chai et al. 2000). Fukamachi et al. (1998) and Wolf et al. (1997) showed that caspase-3 and caspase-9 are very sensitive to changes in the concentration of Zn. Alternatively, Zn can influence other factors that regulate the activation of caspase-3, as in the case of the Bcl-2 protein family and NO. Cui et al. (1999) showed that NO protects murine cells from apoptosis and damage to the intestinal epithelium induced by ZD. Our experimental model of ZD induces the activation of the extrinsic pathway and perhaps the intrinsic apoptosis, a situation which is favored by a strong oxidative and nitrosative stress (Biaggio et al. 2010, 2011; Gomez et al. 2006) and an increase in expression of pro-and anti-apoptotic proteins.

Heat shock proteins Hsp27, Hsp70 and Hsp90 are chaperones whose expression was increased after many different types of stress. Cytoprotective function of Hsps is largely explained by their anti-apoptotic function. Besides, the ability of HSPs to modulate the fate of the cells might have important repercussions in pathological situations. For these reason we analyzed the expression of Hsp27, Hsp70i in lung. Hsp27 and Hsp70i increased significantly in ZD refed group when compared to the control group. Under this context is reasonable to assume that an increase in their expression might modulate the differentiation program and controlled the apoptosis of lung tissue. While Hsp70i expression is inducible in the response to extracellular and intracellular stresses, it exists a constitutively form of Hsp70 which has a role in iNOS induction (Zhang et al. 2013). According to this evidence, we previously reported an increased iNOS activity in our experimental model (Gomez et al. 2006).

Moreover, it was shown that ZD provokes an increase of tumor growth factor-β1 (TGF-β1) protein expression and also induce changes in several proteins of extracellular matrix and filaments intermediates, which are accompanied by a rise of connective tissue (Hagimoto et al. 2002). In point of fact, TGF-β1 is a potent inducer of apoptosis through caspase-3 activation and the downregulation of protein 21 (p21) and is also an enhancer of Fas-mediated apoptosis of lung epithelial cells (Hagimoto et al. 2002). In addition, Li et al. (2005) demonstrated that Hsp27-overexpressing cells prevented morphological alterations of the vimentin filaments, as well as reductions of soluble vimentin, in the cadmium-treated cells. In our experimental model vimentin immunostaining in the ZD group was increased (data not showed) (Biaggio et al. 2011). In conclusion, the cytoprotective properties of Hsp27 result from its ability to modulate ROS. We think that this situation would allow lung remodeling in our ZD refed group.

To summarize, our results provide further evidence of the pathways involved in the relation between cytoprotection and apoptosis and also highlights the critical role that ZD plays in maintaining the lung epithelium and, thereby, preserving the barrier integrity.

A major advantage to this study is our use of whole animal model rather than isolated lung cells. This situation enhanced our ability to infer whether the mechanisms occur in human health and disease. Further studies are required in order to investigate whether Hsp27 and Hsp70 proteins are consistently associated with cellular stress and inflammation in lung pathology caused by ZD. Therefore, it is necessary, to identify, at an early stage, men at risk of lung disease and advice on change of lifestyle and/or Zn supplementation therapy.

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