Dietary zinc deficiency predisposes mice to the development of preneoplastic lesions in chemically-induced hepatocarcinogenesis

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A B S T R A C T

Although there is a concomitance of zinc deficiency and high incidence/mortality for hepatocellular carcinoma in certain human populations, there are no experimental studies investigating the modifying effects of zinc on hepatocarcinogenesis. Thus, we evaluated whether dietary zinc deficiency or supplementation alter the development of hepatocellular preneoplastic lesions (PNL). Therefore, neonatal male Balb/C mice were submitted to a diethylnitrosamine/2-acetylaminefluorene-induced hepatocarcinogenesis model. Moreover, mice were fed adequate (35 mg/kg diet), deficient (3 mg/kg) or supplemented (180 mg/kg) zinc diets. Mice were euthanized at 12 (early time-point) or 24 weeks (late time-point) after introducing the diets. At the early time-point, zinc deficiency decreased Nrf2 protein expression and GSH levels while increased p65 and p53 protein expression and the number of PNL/area. At the late time-point, zinc deficiency also decreased GSH levels while increased liver genotoxicity, cell proliferation into PNL and PNL size. In contrast, zinc supplementation increased antioxidant defense at both time-points but not altered PNL development. Our findings are the first to suggest that zinc deficiency predisposes mice to the PNL development in chemically-induced hepatocarcinogenesis. The decrease of Nrf2/GSH pathway and increase of liver genotoxicity, as well as the increase of p65/cell proliferation, are potential mechanisms to this zinc deficiency-mediated effect.

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1. Introduction

Zinc, an important trace element, is required as a cofactor for approximately 10% of total proteins encoded by the human genome, including enzymes (>200) and transcription factors (>3000) related to basic cellular functions as proper antioxidant defense, immune response, DNA repair and cell proliferation (Andreini et al., 2006; Roohani et al., 2013). According to the US Food and Nutrition Board (2001), the human Recommended Dietary Allowance (RDA) for zinc is 11 and 8 mg/day for adult men and women, respectively. Animal products, mainly sea food and red meat, are the most important dietary sources of readily bioavailable zinc while plant products are considered poor ones (Ma and Betts, 2000).

An estimated 17.3% of the human population is at risk of inadequate zinc intake and subsequent zinc deficiency, mainly in Sub-Saharan Africa and South Asia (Wessells and Brown, 2012). Experimentally, dietary zinc deficiency showed to induce and/or promote chemically-induced esophagus, tongue and colon rodent carcinogenesis (Alder et al., 2012; Christudoss et al., 2012; Fong et al., 2006; Taccioli et al., 2012). In addition, a recent meta-analysis revealed decreased zinc levels in serum and tumor tissue of patients with lung, head and neck, liver, stomach, prostate and thyroid malignant neoplasms (Gumulec et al., 2014). These experimental and human studies indicate a possible underlying role of zinc deficiency on carcinogenesis process. In contrast, zinc supplementation, usually applied to reverse and/or prevent zinc deficiency on human populations (Brooks et al., 2005; Yakoob et al., 2011), showed to inhibit or attenuate chemically-induced colon, forestomach, oral and prostate rodent carcinogenesis (Banudevi et al., 2011; Dani et al.,

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2007; Fong et al., 2011; Sun et al., 2011). Furthermore, zinc supplementation augmented the antitumor effect of adriamycin chemotherapy in MMTV-neu transgenic mouse model of mammary carcinogenesis (Margalit et al., 2012). These *in vivo* studies also suggest a possible role of zinc supplementation on cancer prevention and treatment. In spite of these findings, there are no experimental studies that investigated the modifying effects of zinc deficiency or supplementation on hepatocarcinogenesis.

The Hepatocellular Carcinoma (HCC) is the sixth most common cancer and the second cause of cancer-related deaths worldwide (GLOBOCAN, 2012). The highest incidence and mortality rates due to this malignant neoplasm are found in Asia and Africa (GLOBOCAN, 2012). HCC development is considered a complex, multistep and multifactorial process, involving several risk factors, including chronic hepatitis B and C virus infections, aflatoxin and chronic ethanol intake and non-alcoholic fatty liver disease (NAFLD) (Sanyal et al., 2010).

Mouse models of hepatocarcinogenesis are widely applied in translational research due to morphological and molecular similarities to human HCC (Zucman-Rossi, 2008; Bakiri and Wagner, 2013). Particularly, chemically-induced models provide suitable tools to study several aspects of hepatocarcinogenesis, including risk factors, molecular pathogenesis, prevention and treatment (Bakiri and Wagner, 2013). Diethylnitrosamine (DEN) and 2-acetylaminefluorene (2-AAF) are classical mutagenic/genotoxic agents applied in initiation-promotion models of hepatocarcinogenesis (Park et al., 2011; Santos et al., 2014). Both agents undergo liver metabolism by cytochrome P450 (Jin et al., 2007; Liu et al., 2005), resulting in reactive oxygen species (ROS) and nucleophilic ions, which induce DNA damage and implicate in genomic instability, mutation and ultimately, contribute to the development of hepatocellular preneoplastic and neoplastic lesions (Kraynak et al., 2015; Santos et al., 2014). Notably, the neonatal mouse model of hepatocarcinogenesis is usually established due to the intrinsic susceptibility of neonatal liver to chemical carcinogen exposure, particularly to DEN (Vesselinovitch et al., 1984; Vesselinovitch, 1987). Besides, high cell proliferation rates in the liver of juvenile mice may favor the clonal expansion of initiated hepatocytes, resulting in earlier occurrence of preneoplastic and neoplastic lesions compared to chemically-induced adult mouse models (Vesselinovitch et al., 1984; Vesselinovitch, 1987). In these short- or medium-term bioassays, altered hepatocyte foci (AHF) have been used as putative preneoplastic lesions (PNL) (Pitot, 1990). AHF can be easily identified in liver sections stained by hematoxylin and eosin (HE), which enables the screening of potential causative or preventive factors on early hepatocarcinogenesis, including specific dietary factors (Palmeira et al., 2015; Pitot, 1990), as zinc.

Although there is a concomitance of zinc deficiency and high incidence/mortality for HCC in certain human populations, literature lacks on experimental studies on the modifying effects of zinc deficiency or supplementation on hepatocarcinogenesis process. Thus, we are the first to evaluate whether dietary zinc deficiency or supplementation alter the PNL development in chemically-induced mouse hepatocarcinogenesis.

2. Materials and methods

2.1. Experimental design

Pregnant Balb/C mice were obtained from Multidisciplinary Center for Biological Investigation on Laboratory Animal Science (CEMIB, UNICAMP, Campinas − SP, Brazil). Neonatal male mice were submitted to a classical hepatocarcinogenesis model by receiving a single intraperitoneal (i.p.) injection of DEN [50 mg/kg body weight (b.wt.) in 0.9% saline, Sigma-Aldrich, USA] at postnatal day 15 (PND 15) (Klaunig et al., 1988) (Fig. 1). At PND 28, the mice were randomly allocated into three groups (n = 13 mice each). They were fed AIN-93G semipurified diet (Reeves et al., 1993) containing different concentrations of elemental zinc (Zn) by adding zinc sulphate salt: groups receiving adequate zinc diet (GZnA, 35 mg/kg diet), dietary zinc deficiency (GZnD, 3 mg/kg diet) or dietary zinc supplementation (GZnS, 180 mg/kg diet) (Fig. 1). Also, 2-AAF (Sigma-Aldrich, USA) was incorporated at 0.02% in all experimental diets (Bitsch et al., 2000) (Fig. 1). The animals were euthanized by exsanguination under ketamine/xylazine anesthesia (100/16 mg/kg b.wt., i.p.) at 12 (n = 6 mice/group) or 24 weeks (n = 7 mice/group) after introducing experimental diets (Fig. 1). These endpoints were established in order to evaluate the effects of zinc deficiency or supplementation on early (12 weeks) and late (24 weeks) time-points of hepatocellular PNL development.

Peripheral blood samples were collected from the venous orbital plexus one day before euthanasia to perform Comet Assay for DNA damage analysis. Blood samples were collected by cardiac puncture and serum samples were stored at −20 °C to further determine Alanine Aminotransferase (ALT) levels. At necropsy, the liver was removed, weighed, washed in saline solution (0.9% NaCl) and representative samples from all lobes were collected and processed for histopathological and immunohistochemical analysis. Additional liver samples were collected, snap-frozen in liquid nitrogen and stored at −80 °C to further performance of Comet Assay, western blot, antioxidant profiling and zinc level determination. The animals were kept in a room with ventilation (16−18 air changes/hour), relative humidity (45−65%), controlled temperature (20−24 °C) and light/dark cycle 12:12 and were given deionized water and diet *ad libitum*. Body weight and food consumption were recorded twice a week during the experimental period. The animal experiment was carried out under protocols approved by Botucatu Medical School/UNESP Ethics Committee on Use of Animals (CEUA) (Protocol number 1073/14) and all animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" (2011).

2.2. Diets

Adequate zinc diet was designed to meet the recommended dietary zinc intake for mice (National Academy of Sciences , 2001). Zinc deficient diet was designed to contain ~10-fold lower zinc than adequate diet and to induce low zinc status, similarly to human dietary zinc deficiency, as previously established in chemically-induced carcinogenesis bioassays (Lee et al., 2004; Alder et al., 2012). In contrast, the zinc supplemented diet was produced to contain ~5-fold higher zinc than adequate diet, similarly to rodent studies and usually applied human zinc supplementation regimens (Ko et al., 2005; Huang et al., 2007). Samples of each AIN-93G diet formulation (in triplicate) were submitted to flameless atomic absorption spectroscopy (AAS) analysis (see item 2.7), confirming zinc levels in the experimental diets before introducing them to the mice (adequate zinc: 34.30 ± 4.60; zinc deficiency: 3.40 ± 0.30; zinc supplementation: 174.50 ± 15.80, in mg Zn/Kg diet, values are Mean ± S.D.).

2.3. Histopathological evaluation

Liver samples were fixed in 10% buffered formalin for 24 h at room temperature, stored in 70% ethanol and embedded in paraffin. Five-micron thick liver sections were stained with hematoxilin and eosin (HE). AHF were identified using previously well-established morphological criteria (Thoolen et al., 2010). The incidence of these lesions was calculated for each group. Besides, we also calculated *AHF number/liver area*, by counting all AHF and dividing
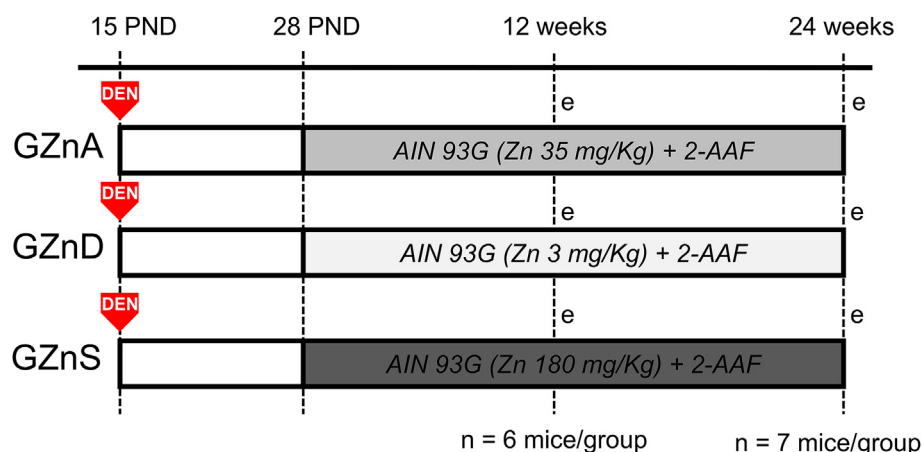
Fig. 1. Experimental design (for details, see Materials and Methods section). PND: postnatal day; Zn: 35, 3 or 180 mg/kg of elemental zinc in AIN-93G diet. DEN: single dose of diethylnitrosamine (50 mg/kg b.w., i.p.); 2-AAF: 2-acetylaminefluorene (0.02% in diet); e: euthanasia; n: number of mice in each euthanasia; GZnA: adequate zinc group; GZnD: zinc deficiency group; GZnS: zinc supplementation group.

by the liver section area analyzed, and *AHF size*, by individually measuring all AHF. Each liver section area (cm^2) and AHF size (mm^2) were measured using KS300 image analysis system (Carl Zeiss, Germany).

2.4. Immunohistochemistry and semi-quantitative analysis

Immunoreactivity for Ki-67 and β-catenin was detected using a universal labeled streptavidin-biotin system (LSAB System-HRP, Dako Cytomation, Denmark). Briefly, deparaffinated 5-μm liver sections on silanized slides were treated sequentially with citrate buffer (120 °C, 5 min) in a Pascal Pressure Chamber (Dako Cytomation, Denmark), 3% H$_2$O$_2$ in phosphate-buffered saline (PBS) (10 min), nonfat milk (60 min), anti-Ki-67 (ab16667, 1:100 dilution, Abcam, UK) or β-catenin (ab32572, 1:400 dilution, Abcam, UK) antibodies overnight (4 °C), followed by biotinylated universal link and streptavidin HPR (20 min each) solutions. Chromogen color development was accomplished with 3,3′-diaminobenzidine tetrahydrochroride (Sigma−Aldrich, USA). The slides were counterstained with Harris's hematoxylin for 1 min. For Ki-67 semi-quantitative analysis in normal-appearing liver tissue, 40 random fields (40× objective) were assessed in sections comprising all liver lobes. Ki-67-positive hepatocytes were counted and divided by the liver area analyzed (mm^2). In AHF, Ki-67-positive hepatocytes were counted and divided by AHF area (mm^2). For β-catenin, only the pattern of staining was evaluated (membranous, cytoplasmic and/ or nuclear) in normal-appearing liver and into AHF. All analyses were performed in an Olympus CellSens software (Olympus Corporation, Japan).

2.5. Comet Assay

Comet Assay was performed on peripheral blood and liver samples by following the well-established protocol proposed by Tice et al. (2000). Peripheral blood samples were collected from the venous orbital plexus one day before euthanasia. Liver samples from the left lobe (~50 mg) were gently minced in ice-cold Ca^{2+} and Mg^{2+} free PBS solution to obtain cell suspensions. Peripheral blood (5 μL) or liver cell suspension (20 μL) were mixed with 100−140 μL low melting point agarose (Invitrogen, USA) dissolved in Ca^{2+} and Mg^{2+} free PBS. The mixture was spread onto microscope slides precoated with normal melting point agarose (Invitrogen, USA). The slides were covered with a coverslip and maintained at 4 °C

(5 min). Then, the coverslip was removed and slides were immersed in cold, freshly prepared, lysis solution containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% N-lauroyl-sarcosine, 1% Triton X-100 and 10% DMSO (all reagents from Sigma-Aldrich, USA) (4 °C, 60 min). After lysis, prior to electrophoresis, slides were placed in a horizontal electrophoresis unit and left in alkaline buffer containing 0.3 mM NaOH and 1 mM EDTA (pH > 13, 20 min) and electrophoresed 30 V (0.86 V/cm) and 300 mA (20 min). Finally, the slides were neutralized in 0.4 M Tris-HCl (pH 7.5), fixed in absolute ethanol and stored at room temperature until blind analysis in a fluorescence microscope (Olympus, Japan) at 20× magnification. Each slide was stained with 80 μL of SYBR® Gold Stain solution (1:10.000, Life Technologies, USA) and immediately analyzed. To minimize extraneous DNA damage from ambient ultraviolet radiation, all steps were performed with reduced illumination. Genotoxicity analysis was performed in Comet Assay IV (Perceptive Instruments, UK). Tail intensity (percentage of DNA in the comet tail) was used as a reliable parameter to evaluate DNA damage in 50 nucleoids *per* slide/two slides *per* animal (Kumaravel and Jha, 2006).

2.6. Hepatic antioxidant profiling and serum ALT

Liver samples were homogenized in 50 mM phosphate buffer (pH 7.4) with auxiliary of the motor−driven teflon glass Potter Elvehjem (100 × g/min). The tissue homogenate was centrifuged (12000 × g, −4 °C, 15 min). The supernatant was used to determine total protein and reduced glutathione (GSH) levels and to evaluate total superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) activities. GSH was measured by a kinetic assay in reaction medium containing 2 mM 5,5′-DTNB acid, 0.2 mM NADPH and 2U of glutathione reductase (Sigma-Aldrich, USA) in phosphate buffer (Sedlak and Lindsay, 1968). SOD activity was determined based on the ability of the enzyme to inhibit the reduction of nitro blue tetrazolium (NBT), which was generated by hydroxylamine in a medium containing phosphate buffer, 0.1 mM EDTA, 50 μM NTB, 78 μM NADH and 33 μM phenazine methosulfate (Ewing and Janero, 1995). Catalase activity was determined in a mixture containing 10 mM hydrogen peroxide and sodium phosphate buffer 50 mM, pH 7.0, in a final volume of 0.3 mL (Bergmeyer, 1974). GPx activity was assayed by following the oxidation of 0.16 mM NADPH in the presence of glutathione reductase which catalyzed the reduction of GSSG formed by the GPx (Nakamura et al., 1974).

Finally, serum ALT levels were determined by a conventional kinetic assay according to the manufacturer's instructions (Liquiform − Labtest Diagnóstica, Brazil). All determinations were performed using a microplate reader (25 °C) (μQuant-Gen5 2.0 software, Bio-Tec Instruments, USA).

2.7. Zinc determination

Liver or AIN-93G diet samples were mineralized in a 16-N nitric acid solution by microwave irradiation (Model DGT 100, Provecto, Brazil). Zinc concentration was determined from the mineralized materials by flameless AAS (GBC, Model 932 AA).

2.8. Western blot

Liver samples were homogenized in lysis buffer (500 nM Tris-HCl, 0.2 M NaCl, 1% Triton X-100, 10 nM $CaCl_2$, and 2 μL/100 mL protease inhibitor cocktail, Sigma-Aldrich, USA) in the proportion of 30 mg of tissue/100 μL of buffer (4 °C, 2 h). After this procedure, the extracted material was centrifuged (4000 rpm, 4 °C, 20 min) and the supernatant collected for protein quantification by Bradford's method. Aliquots of liver homogenates containing 70 μg of total protein were heated (95 °C, 5 min) in sample-loading buffer and then electrophoretically separated in a 12% SDS−PAGE gel under reducing conditions and transferred to nitrocellulose membranes (Sigma-Aldrich, USA). Membranes were blocked with non-fat milk in TBS-T (0.05 M Tris, 0.15 M NaCl, pH 7.2, 1% Tween-20) (1 h). Membranes were subsequently incubated with anti-NFκB p65 (sc-372, 65 KDa, 1:1000 dilution, Santa Cruz Biotechnology, USA), p53 (sc-6243, 53 KDa, 1:500 dilution, Santa Cruz Biotechnology, USA), Nrf2 (ab31163, 68 KDa, 1:1000 dilution, Abcam, UK), β-Catenin (ab32572, 92-86 KDa, 1:5000 dilution, Abcam, UK) or actin (sc1815, 43 KDa, 1:1000 dilution, Santa Cruz Biotechnology, USA) primary antibodies in 5% BSA solution overnight. After 5 wash steps with PBS-T, membranes were incubated with specific horseradish conjugated secondary antibodies, according to the primary antibodies used (2 h). Finally, after 5 wash steps, the membranes were submitted to immunoreactive protein signals detected using the Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare Life Sciences, UK). Signals were captured by a G:BOX Chemi system (Syngene, UK) controlled by an automatic software (GeneSys, Syngene, UK). Band intensities were quantified using densitometry analysis software (Image J software, Austria) and p53, NFκB (p65), Nrf2 and β-catenin protein expression was reported as fold change according to actin protein expression, used as a normalizer. For each marker (in each time-point), we ran 2 samples of each group/gel, totalizing 3 gels and 6 samples/group.

2.9. Statistical analysis

Data were analyzed by One-Way ANOVA and post hoc Tukey's test. The incidence of histopathological findings was analyzed by Fisher's Exact Test. The differences were considered significant at $p < 0.05$. All statistical analyses were performed using 3.5 Jandel Sigma Stat software (Jandel Corporation, USA).

3. Results

3.1. Zinc deficiency and supplementation do not alter food consumption, liver and body weight

During the whole experimental period, no death or specific clinical signs were observed. Moreover, final body weight, body weight gain, food intake and relative liver weight (%) values were similar among the experimental groups at both early (week 12) and

Table 1
Effects of dietary zinc deficiency or supplementation on food consumption, liver and body weights, and hepatic zinc levels at weeks 12 and 24.

Period/Parameters	Groups[a]		
	GZnA	GZnD	GZnS
PND 28 − Week 12 (n = 6 mice/group)			
Final body weight (g)	26.7 ± 1.36	29.6 ± 1.50	26.6 ± 1.36
Body weight gain (g)	11.6 ± 2.06	12.6 ± 2.51	13.0 ± 1.89
Liver relative weight (%)	4.15 ± 0.30	4.43 ± 0.21	4.67 ± 0.11
Food consumption (g/mouse/day)	3.31 ± 0.16	3.35 ± 0.43	3.29 ± 0.30
Liver Zinc (μg/g tissue)	25.1 ± 0.47	24.3 ± 0.32	26.0 ± 0.87
PND 28 − Week 24 (n = 7 mice/group)			
Final body weight (g)	31.0 ± 2.16	32.8 ± 4.77	29.8 ± 0.89
Body weight gain (g)	17.0 ± 3.26	15.0 ± 4.65	15.0 ± 2.58
Liver relative weight (%)	4.95 ± 0.95	4.27 ± 0.78	4.24 ± 0.89
Food consumption (g/mouse/day)	3.52 ± 0.32	3.78 ± 1.33	3.41 ± 0.31
Liver Zinc (μg/g tissue)	26.8 ± 1.08	22.5 ± 1.43*	25.3 ± 4.75

Values are Mean ± S.D.
[a] GZnA (adequate zinc, 35 mg/kg diet), GZnD (zinc deficiency, 3 mg/kg diet) or GZnS (zinc supplementation, 180 mg/kg diet) from postnatal day 28 until weeks 12 or 24. All mice were submitted to DEN/2-AAF treatment (see Material and Methods section). Asterisks correspond to statistical difference compared to GZnA group by ANOVA *post hoc* Tukey's test: *$p < 0.01$.

late (week 24) time-points (Table 1). Some studies reported decreased appetite and body weight in rats exposed to dietary zinc deficiency (Lee et al., 2004; Song et al., 2009). However, these alterations are not frequently observed in mice, as previously reported by others (Zhong et al., 2013).

### 3.2. Zinc deficiency decreases hepatic zinc levels

In fact, dietary zinc deficiency decreased hepatic zinc levels in comparison to adequate zinc diet at week 24 ($p = 0.006$) (Table 1), as previously reported (Alder et al., 2012; Zhong et al., 2013). However, dietary zinc supplementation did not significantly alter hepatic zinc levels. Indeed, increased hepatic zinc concentrations are only observed in higher dietary zinc supplementation regimens (300 mg/kg) (Jing et al., 2015).

3.3. Zinc deficiency increases the number of AHF/liver area and AHF size

Histopathological examination revealed PNL showing basophilic, eosinophilic or clear cells (Fig. 2A). The groups did not significantly differ on the incidence of the different types of PNL in both experimental periods (Table 2). However, dietary zinc deficiency increased the number of AHF *per* liver area at the early (week 12) and AHF size at the late time-point (week 24) ($p = 0.004$ and $p < 0.001$, respectively) in comparison to the other groups (Fig. 2B and C). ALT serum levels were similar among the experimental groups at week 12 (GZnA: 52.83 ± 11.14; GZnD: 59.89 ± 13.26; GZnS: 61.63 ± 9.65, in U/L, values are Mean ± S.D.) and week 24 (GZnA: 64.57 ± 14.38; GZnD: 57.86 ± 13.31; GZnS: 59.87 ± 9.65, in U/L, values are Mean ± S.D.).

3.4. Zinc deficiency increases cell proliferation into AHF

Ki-67 immunoreactivity revealed typical nuclear staining in hepatocytes of normal-appearing liver tissue and into AHF (Fig. 3A). AHF containing Ki-67-positive hepatocytes were only observed at the late time-point (week 24). Ki-67 semi-quantitative analysis in normal-appearing liver areas was similar among the experimental groups at both moments (Fig. 3B). Nonetheless, zinc deficiency increased cell proliferation in preneoplastic AHF in comparison to the other groups at week 24 ($p = 0.016$) (Fig. 3B). Lastly, β-catenin
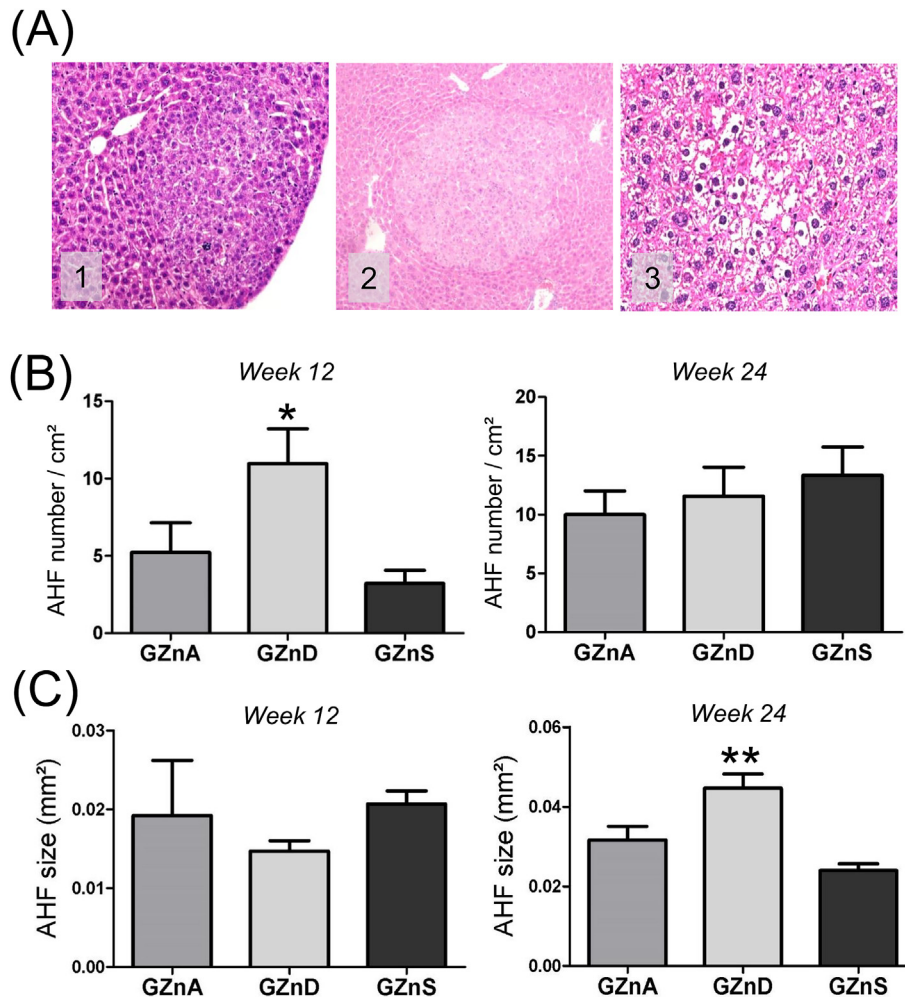
Fig. 2. (A) Representative photomicrographs of AHF showing *(1)* basophilic (20× objective), *(2)* eosinophilic (20× objective) and *(3)* clear cells (40× objective) in HE-stained sections. Effects dietary of zinc deficiency or supplementation on **(B)** AHF number *per* liver area (cm^2) and **(C)** AHF size (mm^2). Values are Mean ± S.D. GZnA (adequate zinc, 35 mg/kg diet), GZnD (zinc deficiency, 3 mg/kg diet) or GZnS (zinc supplementation, 180 mg/kg diet) from postnatal day 28 until week 12 (n = 6 mice/group) or 24 (n = 7 mice/group). All groups were submitted to DEN/2-AAF treatment (see Material and Methods section). AHF = altered hepatocyte foci. Asterisks correspond to statistical difference compared to the other groups by ANOVA *post hoc* Tukey's test: *p < 0.01; **p < 0.001.

Table 2
Effects of dietary zinc deficiency or supplementation on the incidence of preneoplastic AHF at weeks 12 and 24.

Week/AHF type	Groups[a]		
	GZnA	GZnD	GZnS
Week 12 (n = 6 mice/group)			
Basophilic cell foci	5/6 (83.3%)	6/6 (100%)	6/6 (100%)
Eosinophilic cell foci	0/6 (0)	0/6 (0)	0/6 (0)
Clear cell foci	0/6 (0)	0/6 (0)	0/6 (0)
Week 24 (n = 7 mice/group)			
Basophilic cell foci	4/7 (57.1%)	4/7 (57.1%)	4/7 (57.1%)
Eosinophilic cell foci	7/7 (100%)	6/7 (85.7%)	7/7 (100%)
Clear cell foci	1/7 (14.3%)	0/7 (0)	1/7 (14.3%)

Data represent the proportion of affected animals (percentage) and were analyzed by Fisher's Exact Test (p < 0.05).
[a] GZnA (adequate zinc, 35 mg/kg diet), GZnD (zinc deficiency, 3 mg/kg diet) or GZnS (zinc supplementation, 180 mg/kg diet) from postnatal day 28 until weeks 12 or 24. All groups were submitted to DEN/2-AAF treatment (see Material and Methods section). AHF = Altered Hepatocyte Foci.

analysis showed typical membranous staining (Fig. 3A) in normal-appearing liver areas and AHF in all mice from all experimental groups.

3.5. Zinc deficiency decreases GSH levels and increases peripheral blood and liver genotoxicity

Although zinc does not function as an antioxidant *per se*, this metal is essential for multiple enzymes participating in antioxidant defense (Kloubert and Rink, 2015). Dietary zinc deficiency decreased hepatic GSH levels when compared to adequate zinc diet and zinc deficiency at both moments (weeks 12 and 24) (p < 0.001, for both) (Table 3). Moreover, zinc deficiency increased 2-AAF-induced genotoxicity in both peripheral blood and liver cells when compared to the other groups at the late time-point (week 24) (p = 0.037 and p = 0.023, respectively) (Fig. 4).

3.6. Zinc supplementation increases antioxidant defense and decreases peripheral blood genotoxicity

Dietary zinc supplementation increased hepatic GSH levels and GPx activity when compared to adequate zinc diet and zinc deficiency at week 12 (p < 0.001, for both) and week 24 (p < 0.001, for both) (Table 3). Zinc supplementation also increased total SOD and catalase activities in comparison to the other groups only at week 24 (p < 0.001 and p = 0.038, respectively). In addition, zinc
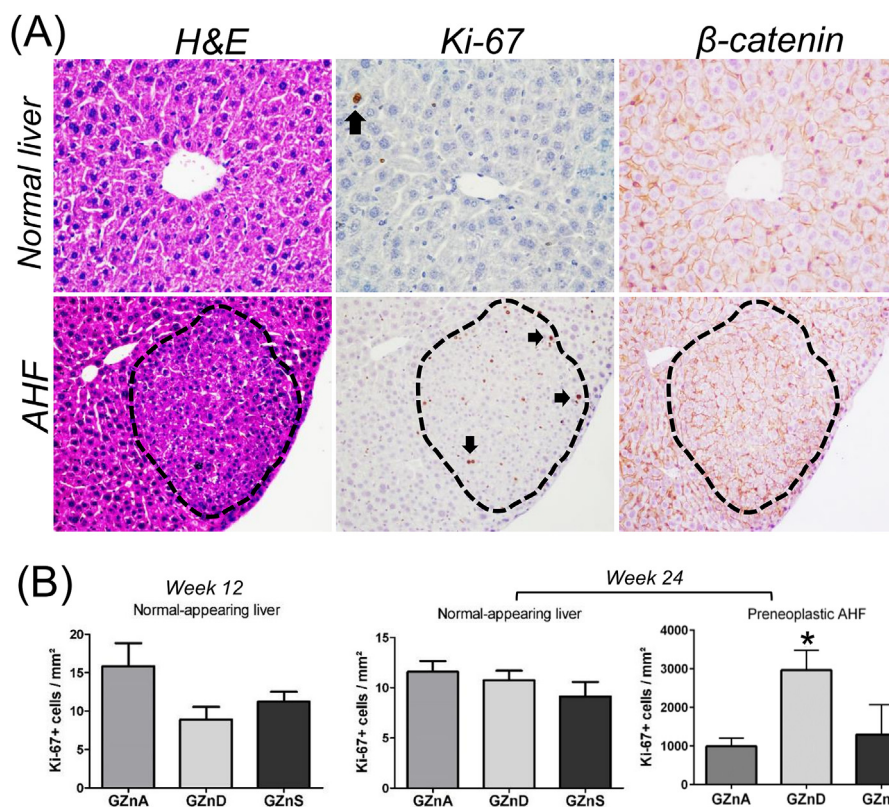
Fig. 3. (A) Representative photomicrographs of liver HE staining and respective nuclear Ki-67 (arrows) and membranous β-catenin staining in normal-appearing liver (40×
objective) and basophilic AHF (20× objective). (B) Effects of dietary zinc deficiency or supplementation on cell proliferation (Ki-67) at weeks 12 and 24. Values are Mean ± S.D. GZnA
(adequate zinc, 35 mg/kg diet), GZnD (zinc deficiency, 3 mg/kg diet) or GZnS (zinc supplementation, 180 mg/kg diet) from postnatal day 28 until week 12 (n = 6 mice/group) or 24
(n = 7 mice/group). All groups were submitted to DEN/2-AAF treatment (see Material and Methods section). AHF = Altered hepatocyte foci. Asterisks correspond to statistical
difference compared to the other groups by ANOVA *post hoc* Tukey's test: *p < 0.05.

Table 3
Effects of dietary zinc deficiency or supplementation on liver antioxidant profiling at weeks 12 and 24.

Week/Parameters	Groups[a]		
	GZnA	GZnD	GZnS
Week 12 (n = 6 mice/group)			
GSH (nmol/g tissue)	8.03 ± 0.72	6.15 ± 0.64**	10.8 ± 1.25**
SOD (nmol/mg protein)	40.7 ± 5.12	42.4 ± 6.01	45.5 ± 9.25
Catalase (μmol/g tissue)	19.5 ± 1.59	17.6 ± 2.77	19.9 ± 1.85
GPx (nmol/mg tissue)	11.6 ± 1.57	10.7 ± 1.48	15.1 ± 1.48**
Week 24 (n = 7 mice/group)			
GSH (nmol/g tissue)	7.49 ± 1.17	5.22 ± 0.68**	8.86 ± 0.52**
SOD (nmol/mg protein)	41.9 ± 5.86	37.3 ± 3.24	51.5 ± 7.26**
Catalase (μmol/g tissue)	23.8 ± 7.83	23.4 ± 10.5	32.6 ± 7.75*
GPx (nmol/mg tissue)	8.93 ± 1.44	10.6 ± 1.09	20.5 ± 2.16**

Values are Mean ± S.D.
[a] GZnA (adequate zinc, 35 mg/kg diet), GZnD (zinc deficiency, 3 mg/kg diet) or GZnS (zinc supplementation, 180 mg/kg diet) from postnatal day 28 until weeks 12 or 24. All mice were submitted to DEN/2-AAF treatment (see Material and Methods section). GSH = reduced glutathione; SOD = superoxide dismutase; GPx = glutathione peroxidase. Asterisks correspond to statistical difference compared to the other groups by ANOVA *post hoc* Tukey's test: *p < 0.05; **p < 0.001.

supplementation reduced 2-AAF-induced genotoxicity in periph-
eral blood cells when compared to adequate zinc diet at week 12
(p = 0.032) (Fig. 4).

### 3.7. Zinc deficiency decreases Nrf2 and increases p53 and p65 protein expression

Since alterations in p53, p65 and β-catenin pathways are

frequently observed in both human and rodent chemically-induced
hepatocarcinogenesis (Jenkins and Parry, 2000; Anna et al., 2003;
Park et al., 2005; Hussain et al., 2007; Majumder et al., 2010;
Luedde and Schwabe, 2011), we investigated the effects of zinc
deficiency or supplementation on the protein expression of these
markers. At the early time-point (week 12), dietary zinc deficiency
increased hepatic NFκB (p65) and p53 protein expression when
compared to zinc supplementation and adequate zinc diet
(p = 0.007 and p = 0.002, respectively) (Fig. 5). In contrast, dietary
zinc supplementation decreased hepatic β-catenin protein
expression when compared to adequate zinc diet (p = 0.033)
(Fig. 5).

We also evaluated Nrf2 protein expression, since alterations in
Keap1/Nrf2/ARE pathway are common during the early stages of
DEN/2-AAF-induced rodent hepatocarcinogenesis (Zavattari et al.,
2015). Dietary zinc deficiency decreased hepatic Nrf2 protein
expression in comparison to adequate zinc diet (p = 0.026) at the
early time-point (week 12) (Fig. 5). Groups showed similar protein
expression of these markers at the late time-point (week 24).

4. Discussion

The present study investigated the modifying effects of dietary
zinc deficiency or supplementation on the development of PNL in
chemically−induced mouse hepatocarcinogenesis. At the early
time-point (12 weeks), zinc deficiency decreased Nrf2 protein
expression and GSH levels while increased p65 and p53 protein
expression and the number of preneoplastic AHF/liver area. At the
late time-point (24 weeks), zinc deficiency also decreased GSH

Peripheral blood　　　　　　　　　　**Liver**
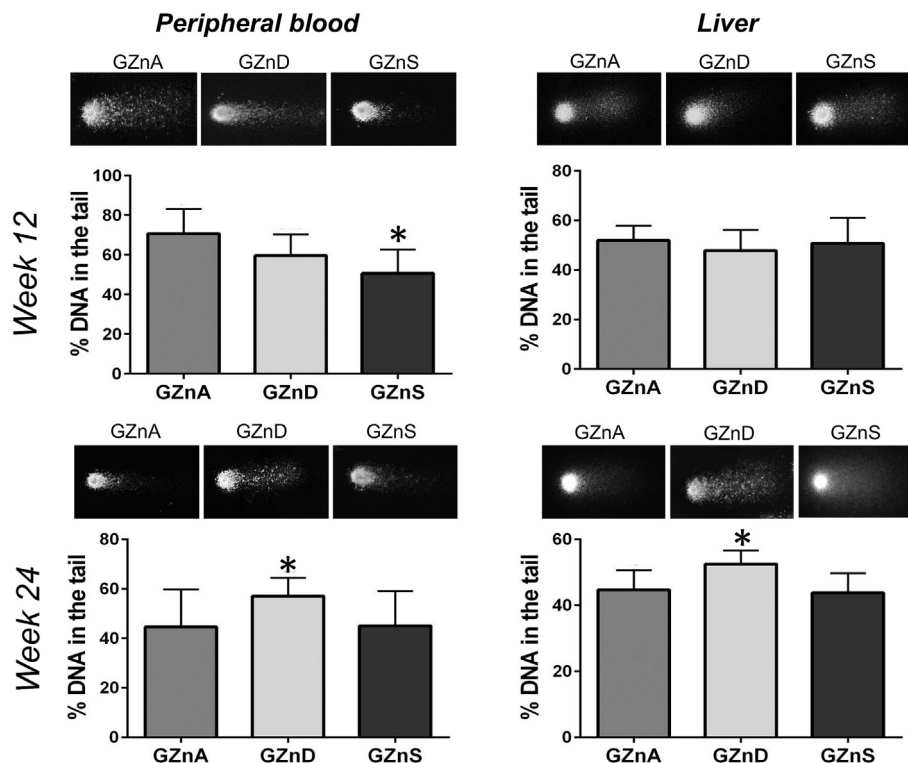
Fig. 4. Effects of dietary zinc deficiency or supplementation on 2-AAF-induced genotoxicity in peripheral blood and liver at weeks 12 and 24. Representative photomicrographs of comet patterns are also presented (40× objective). Values are Mean ± S.D. GZnA (adequate zinc, 35 mg/kg diet), GZnD (zinc deficiency, 3 mg/kg diet) or GZnS (zinc supplementation, 180 mg/kg diet) from postnatal day 28 until weeks 12 (n = 6 mice/group) or 24 (n = 7 mice/group). All mice were submitted to DEN/2-AAF treatment (see Material and Methods section). Asterisks correspond to statistical difference compared to GZnA or to all groups by ANOVA *post hoc* Tukey's test: *p < 0.05.

levels while increased liver and peripheral blood genotoxicity, cell proliferation into AHF and AHF size.

As highlighted before, zinc has essential roles in antioxidant defense (Kloubert and Rink, 2015). Especially, the Keap1/Nrf2/ARE pathway is the main regulator of antioxidant/cytoprotective responses to endogenous and exogenous stresses caused by ROS and electrophiles (Nguyen et al., 2009). Under basal conditions, Keap1 dimer binds Nrf2 protein, leading it to proteasomal degradation (Nguyen et al., 2009). Under stressful conditions, Keap1 is inhibited, allowing Nrf2 protein to accumulate and to bind the antioxidant response element (ARE) (Nguyen et al., 2009). Finally, the Nrf2/ARE complex activates the expression of genes related to GSH synthesis, as glutamate–cysteine ligase (GCL) enzyme (Lu, 2013). GSH, a potent electron-donator, can directly interact with ROS and electrophiles, providing protection against electrophilic DNA-damaging agents (Ballatori et al., 2009), such as DEN and 2-AAF.

The Keap1/Nrf2/ARE pathway perceives stress partly by zinc signaling (McMahon et al., 2010). Stressful conditions increase intracellular free zinc (Zn^{2+}) levels by the oxidation and degradation of zinc-containing proteins (Kröncke, 2007). Keap1 holds domains that can act as "zinc sensors": enhanced intracellular zinc levels may act as negative regulators of Keap1, releasing and allowing Nrf2 to accumulate in cell (McMahon et al., 2010). In the present study, considering the importance of zinc on Keap1/Nrf2/ARE pathway activation, dietary zinc deficiency decreased Nrf2 protein expression at week 12. This decrease at the early time-point reduced GSH levels at both time-points (weeks 12 and 24) and, ultimately, contributed to enhance carcinogen (2-AAF)-induced oxidative stress and genotoxicity in the peripheral blood and especially in the liver at the late time-point (week 24) (Fig. 6). Similar findings were reported by recent *in vitro* studies (Omata

et al., 2013; Li et al., 2014).

Zinc is also related to DNA repair since the DNA-binding domain of p53, a protein that coordinates proper DNA repair, exhibits a complex tertiary structure stabilized by zinc atoms (Loh, 2010; Méplan et al., 2000). Under hypoxia and/or oxidative stress, the p53 pathway is activated: p53 accumulates in the cell, leading to the expression of genes related to DNA repair, as *GADD45* (Riley et al., 2008). In the present study, dietary zinc deficiency increased hepatic p53 protein expression at week 12. In previous studies, zinc depletion also enhanced p53 protein expression in human lung fibroblasts and glioma cells. However, the p53 ability to bind target DNA sequences was reduced (Ho and Ames, 2002; Ho et al., 2003). Decreased intracellular zinc levels induce structural changes in p53 DNA-binding domain, impairing p53 DNA-binding capacity and proper DNA repair (Méplan et al., 2000; Verhaegh et al., 1998). Although we observed an increase in p53 expression at the early time-point, possibly as a result of enhanced carcinogen-induced oxidative stress caused by zinc deficiency, this protein may be structurally changed, and hence DNA repair is impaired. Thus, dietary zinc deficiency may not only impair antioxidant mechanisms as Nrf2/GSH, but also DNA repair mechanisms mediated by p53. As a consequence, these early alterations (week 12) may have contributed to augment carcinogen (2-AAF) induced-DNA damage at the late time-point (week 24) (Fig. 6).

In the early stage of chemically-induced hepatocarcinogenesis, the drastically modified hepatic environment of increased oxidative stress and DNA damage favors the emerging of preneoplastic AHF, possibly from the clonal expansion of initiated hepatocytes (Ogawa, 2009). Under stimuli, these lesions accumulate several molecular alterations and acquire phenotypic characteristics as genomic instability and high cell proliferation, which contribute to
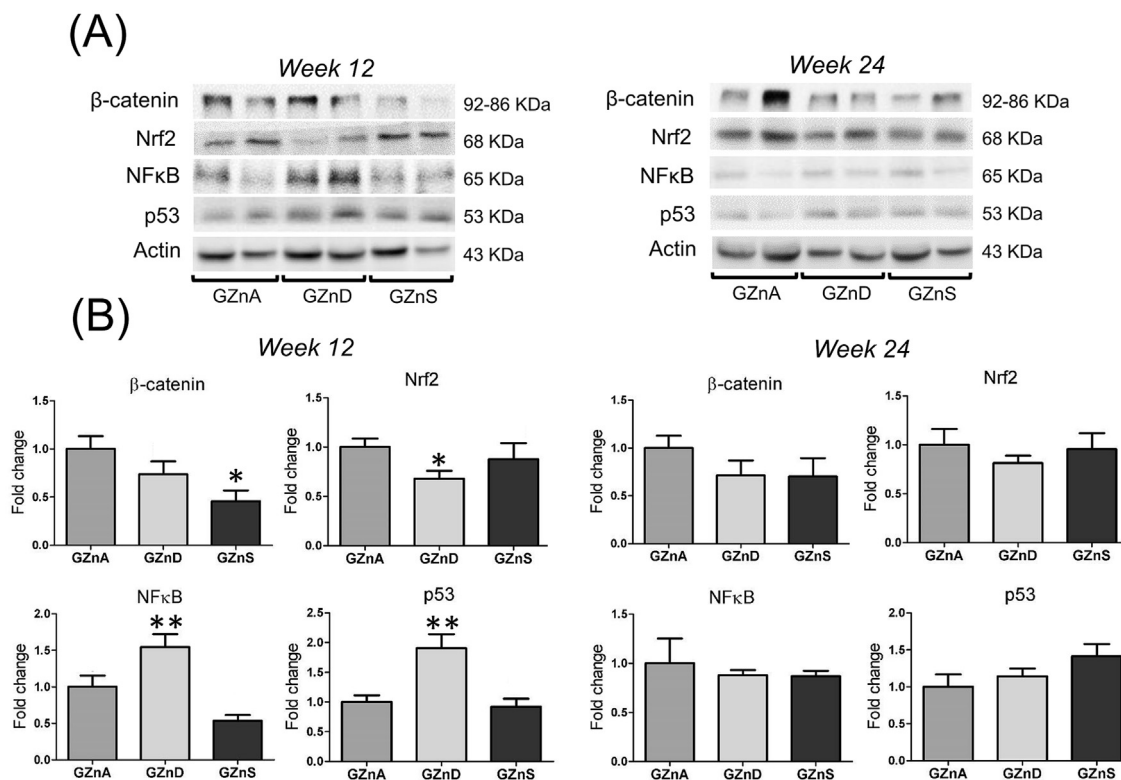
Fig. 5. Effects of dietary zinc deficiency or supplementation on hepatic β-catenin, Nrf2, NFκB and p53 protein expression at weeks 12 and 24. **(A)** Representative blot bands and **(B)** Semi-quantitative analysis by densitometry. Values are Mean ± S.D. GZnA (adequate zinc, 35 mg/kg diet), GZnD (zinc deficiency, 3 mg/kg diet) or GZnS (zinc supplementation, 180 mg/kg diet) from postnatal day 28 until week 12 (n = 6 mice/group) or 24 (n = 7 mice/group). All mice were submitted to DEN/2-AAF treatment (see Material and Methods section). Asterisks correspond to statistical difference compared to GZnA or to all groups by ANOVA *post hoc* Tukey's test: *$p < 0.05$; **$p < 0.01$.
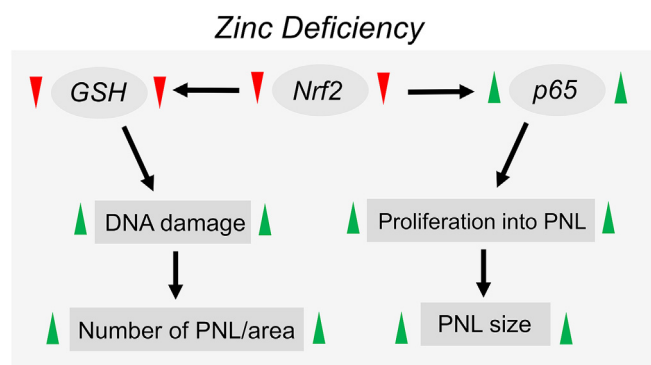
Fig. 6. Schematics of the predisposing effects of zinc deficiency on the development of hepatocellular preneoplastic lesions (PNL). The decrease of Nrf2/GSH pathway and increase of liver genotoxicity, as well as the increase of p65/cell proliferation, are potential mechanisms to this zinc deficiency-mediated effect. GSH = reduced glutathione; ▲ = increase; ▼ = decrease.

AHF development and ultimately, to AHF progression to adenomas and HCC (Ogawa, 2009). In the present study, the hepatic environment of defective antioxidant defense (Nrf2/GSH), DNA repair (p53) and enhanced DNA damage provided by zinc deficiency contributed to PNL development, resulting in enhanced number of AHF *per* liver area at week 12 (Fig. 6).

The nuclear factor κB (NFκB) is a redox-sensitive transcription factor present in the cytoplasm as an inactive heterotrimer (p50, p65, and IkB subunits) (Morgan and Liu, 2011). Under oxidative stress and other stimuli, IkB is phosphorylated by IκB quinase (IKKβ), leading to nuclear translocation of p50 and p65 subunits

and enhanced expression of genes involved in cell proliferation (as *COX-2* and *cyclin D1*) (Morgan and Liu, 2011). Additionally, studies already reported a crosstalk between Nrf2 and NFκB pathways, usually inversely correlated: when Nrf2 pathway is activated, Keap1 acts as a negative regulator of NFκB signaling through the inhibition and degradation of IKKβ (Kim et al., 2010; Lee et al., 2009).

Therefore, the reduction of Nrf2 protein expression mediated by zinc deficiency at the early time-point (week 12) has provided the stimulus for NFκB pathway activation at the same time-point. When activated, the NFκB (p65) transcription factor augmented the cell proliferation into preneoplastic AHF and, as a result, AHF size at the late time-point (week 24) (Fig. 6). In agreement, Majumder et al. (2010) observed that zinc-dependent metallothionein knockout induced oxidative stress, NFκB pathway activation, followed by an increase in cell proliferation and in the incidence of hepatocellular adenoma/carcinoma in a DEN-induced mouse hepatocarcinogenesis. Increased cell proliferation into AHF benefits the clonal expansion of DEN/2-AAF initiated hepatocytes and favors the accumulation of molecular alterations that predispose to AHF growth and progression to tumors (Ogawa, 2009). Then, the increases in the number of AHF *per* liver area, AHF cell proliferation and, consequently, AHF size may be considered direct predisposing effects of zinc deficiency on chemically-induced hepatocarcinogenesis.

In contrast, dietary zinc supplementation enhanced total SOD and catalase activities at week 24 and increased hepatic GPx activity and GSH levels at both time-points. Zinc is an essential structural component of copper and zinc-dependent superoxide dismutase enzyme (CuZnSOD) (Zelko et al., 2002). Also, zinc atoms are part of zinc finger proteins responsible for DNA-binding activity

of Sp1 transcription factor which induces the expression of catalase, another important antioxidant enzyme (Lee et al., 2005). Thus, increased levels or activity of these antioxidant agents may have protected peripheral blood cells against ROS and nucleophilic ions produced by DEN/2-AAF metabolism, resulting in decreased DNA damage at week 12.

Although zinc supplementation (drinking water, 75 mg/L) already showed to attenuate mouse alcoholic hepatic injury by inhibiting the generation of ROS and enhancing antioxidant pathways (Zhou et al., 2005), dietary zinc supplementation used herein did not alter the incidence or the number of preneoplastic AHF *per* liver area, as well as AHF size and cell proliferation. On the other hand, zinc supplementation reduced β-catenin protein expression at the early time-point (week 12). The Wnt/β-catenin pathway plays important roles in liver growth, regeneration and proliferation (Micsenyi et al., 2004; Tan et al., 2006). In contrast, Wnt/β-catenin pathway aberrant activation, as a result of mutations in β-catenin gene, is frequently found in human and chemically-induced HCC, including DEN-induced models (Anna et al., 2003; Park et al., 2005). Therefore, it is hypothesized that increased antioxidant defense as well as decreased β-catenin protein expression could provide a hepatoprotective environment against late HCC development. However, further long-term studies are necessary.

Taken together, our findings demonstrate that dietary zinc deficiency predisposes mice to the development of PNL in chemically-induced hepatocarcinogenesis. The decrease of Nrf2/GSH pathway and subsequent increase of liver genotoxicity, as well as the increase of p65/cell proliferation, are proposed as potential mechanisms to this zinc deficiency-mediated effect (Fig. 6). Our findings could also indicate an underlying role of this nutritional imbalance as a risk cofactor of human hepatocarcinogenesis. To our knowledge, this is the first report of the predisposing effects of dietary zinc deficiency on a classical and well-established hepatocarcinogenesis model. In contrast, despite of conferring a hepatoprotective environment by enhancing antioxidant defense, dietary zinc supplementation does not protect mice against the development of PNL in chemically-induced hepatocarcinogenesis.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2016.08.020.

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