Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry 44 (2017) 71–79

Journal of Nutritional Biochemistry

Early-in-life dietary zinc deficiency and supplementation and mammary tumor development in adulthood female rats

Flávia R.M. da Silva[a], Tony F. Grassi[b], Joyce R. Zapaterini[a], Lucas T. Bidinotto[c,d], Luis F. Barbisan[b,*]

[a]UNESP – Univ. Estadual Paulista, School of Medicine, Department of Pathology, Botucatu, São Paulo-SP, Brazil
[b]UNESP – Univ. Estadual Paulista, Institute of Biosciences, Department of Morphology, Botucatu, São Paulo-SP, Brazil
[c]Molecular Oncology Research Center, Barretos Cancer Hospital, Barretos, SP, Brazil
[d]Barretos School of Health Sciences, Dr. Paulo Prata - FACISB, Barretos, SP, Brazil

Received 20 September 2016; received in revised form 16 December 2016; accepted 2 March 2017

Abstract

Zinc deficiency during pregnancy and postnatal life can adversely increase risk of developing human diseases at adulthood. The present study was designed to evaluate whether dietary zinc deficiency or supplementation during the pregnancy, lactation and juvenile stages interferes in the development of mammary tumors induced by 7,12-dimethylbenzanthracene (DMBA) in female Sprague–Dawley (SD) rats. Pregnant female SD rats were allocated into three groups: zinc-adequate diet (ZnA - 35-mg/kg chow), zinc-deficient diet (ZnD - 3-mg/kg chow) or zinc-supplemented diet (ZnS - 180-mg/kg chow) during gestational day 10 (GD 10) until the litters' weaning. Female offspring received the same diets as their dams until postnatal day (PND) 51. At PND 51, the animals received a single dose of DMBA (50 mg/kg, ig) and zinc-adequate diets. At PND 180, female were euthanized, and tumor samples were processed for histological evaluation and gene expression microarray analysis. The ZnD induced a significant reduction in female offspring body weight evolution and in mammary gland development. At late in life, the ZnD or ZnS did not alter the latency, incidence, multiplicity, volume or histological types of mammary tumors in relation to the ZnA group. However, the total tumor number in ZnS group was higher than in ZnA group, accompanied by distinct expression of 4 genes up- and 15 genes down-regulated. The present findings indicate that early-in-life dietary zinc supplementation, differently to zinc deficiency, has a potential to modify the susceptibility to the development of mammary tumors induced by DMBA.

Keywords: Dietary zinc status; Prenatal and postnatal life; Rat mammary tumors; Adulthood; Female Sprague-Dawley rats

1. Introduction

Zinc (Zn) is the most common trace element in eukaryote cells and the second most abundant trace element in human tissues and secretions [1]. It is essential for the activity of more than 300 enzymes which are involved in a number of cellular processes such as cell differentiation, growth, transcription, cell signaling, proliferation, apoptosis and immunological responses [1–3]. Some main human dietary sources of Zn include red meat, poultry, fish and other seafood, legumes, nuts, whole grains, and dairy products [4–5].

Several studies have investigated the relationship between tissue zinc level and the risk for the development of different types of cancer [6–10]. In general, in high serum concentrations, zinc is associated to reducing risk of tumor development, while serum zinc-deficient levels have been associated to cancer progression and malignancy, but these findings are still not consistent [8–10]. In addition, several types of malignant human tissues have showed lower zinc concentrations when compared to their normal counterparts [6–10]. It is still not well established whether this altered zinc distribution in serum and cancerous tissues is a contributing factor or a consequence of the tumor development itself (*i.e.*, chronic oxidative stress or altered zinc nutrition) [6–10].

Breast cancer is a chronic disease with endogenous and environmental etiology, and it is considered as a heterogeneous disease [11]. In fact, trace elements or metals have been implicated in the pathogenesis of this malignancy [6,12]. The paradox of zinc biological effects is especially prevalent in mammary carcinogenesis [12]. Zinc concentrations have been reported to be significantly higher in breast cancer tumors compared to healthy breast tissue and lower in blood serum and erythrocytes of breast cancer patients when compared to healthy controls [6,13–15]. It is possible that breast cancer cells uptake more zinc during uncontrolled growth, and thus, it may be necessary to have more zinc plasmatic replenishment [16]. Moreover some studies also show an inverse relationship between plasmatic zinc levels and the risk of breast cancer development [15,16], suggesting that low zinc levels may be prognostic and therapeutic factors for this disease development/progression [15].

* Corresponding author at: Departamento de Morfologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Rua Prof. Dr. Antonio Celso Wagner Zanin s/n, 18618-689, Botucatu, SP, Brazil. Tel./fax: +55-14-38800469.
E-mail address: barbisan@ibb.unesp.br (L.F. Barbisan).

http://dx.doi.org/10.1016/j.jnutbio.2017.03.003
0955-2863/

Low-zinc dietary status during postinitiation phase has showed a suppressive effect on N-methyl-N-nitrosourea (MNU)-induced rat mammary tumorigenesis in adult female SD rats [16]. In this study, plasmatic zinc concentration, tumor incidence, tumor multiplicity and total number of tumors were lower in low-zinc group than in adequate-zinc *ad libitum* and pair-fed control groups. In a previous study, it was observed that zinc accumulation in mammary tumors induced by MNU in female SD rat was associated with an increased mRNA level of *ZnT-1* (zinc transporter efflux) and increased mRNA and protein levels of metallothionein when compared with normal mammary gland [17]. However, there are some divergences about zinc concentration in mammary carcinogenesis [12]. *In vitro* and *in vivo* studies have revealed that zinc deficiency leads to an increased oxidative stress and DNA damage, which contributed to cancer development [3,6,18–20], whereas zinc supplementation has been shown to inhibit this disease [2,19].

A recent experimental study showed that intake of marginal zinc-deficient diet (15-mg Zn/kg chow) by adult female C57/Bl6 mice resulted in high zinc accumulation and zinc transporters *ZIP-6* and *ZIP-10* over-expression in mammary tissue [20]. These alterations lead to ductal and stromal inflammation, fibrosis, oxidative stress, mammary gland expansion and increase of estrogen receptor (ER) expression. Thus, marginal zinc deficiency intake in adulthood can also induce zinc accumulation and oxidative stress in the mammary tissue [20]. Other experimental studies have showed that moderate-to-severe zinc deficiency during fetal life and lactation has shown adverse effects on rat blood pressure and renal and cardiac function in adult life [21–23].

To our knowledge, there are no experimental studies of early-in-life dietary zinc status on late effects on chemically induced mammary carcinogenesis. Thus, the aim of the present study was to investigate the influence of dietary zinc deficiency and zinc supplementation during early life and their effects on adulthood mammary gland of female Sprague–Dawley rats initiated with DMBA.

2. Material and methods

2.1. Chemical and diets

7,12-Dimethylbenzantracene (DMBA) was purchased from Sigma Chemical Co. (CAS 57–97-6, ~ 95%: - St. Louis, MO, USA). The semipurified diets (AIN-93G) were obtained from HN&C – Consulting in Experimental Nutrition (Campinas-SP, Brazil) with different zinc levels (zinc sulfate): zinc-adequate diet (ZnA, 35-mg/kg chow); zinc-deficient diet (ZnD, 3-mg/kg chow) and zinc-supplemented diet (ZnS180-mg/kg chow) [24]. Samples of each AIN-93G diet formulation (in triplicate) were submitted to flameless atomic absorption spectroscopy (AAS) analysis, confirming zinc levels in the experimental diets before introducing them to the dams [24].

2.2. Animals and treatments

The animal protocols used in this study were consistent with Ethical Principles for Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the School of Medicine/UNESP Ethical Committee for Animal Research (CEEA, protocol number 917).

Female (n=60) and male (n=30) Sprague–Dawley (SD) rats, 4-week old, were obtained from Multidisciplinary Center for Biological Research (CEMIB, UNICAMP, Campinas - SP, Brazil). The animals were allocated into polypropylene cages containing laboratory-grade pine shavings as bedding and maintained in a room under controlled temperature (22±2 °C), relative humidity (55±10%), lighting conditions (12-h light/12-h dark photoperiod) and continuous exhaust air. Adult female rats (12-week old) in proestrus were mated 2:1 with males in the cage for 12 h (dark period). The presence of sperm in the vaginal smear and identification of the estrus phase of the estrous cycle was considered the gestational day 0 (GD0). These procedures were performed until we obtain the necessary number of pregnant females to each experimental group. The pregnant female rats (16 dams/group) were maintained in individual cages and were randomly distributed into three groups: ZnA, dams fed zinc-adequate diet; ZnD, dams fed zinc-deficient diet; and ZnS, dams fed zinc-supplemented diet. These experimental diets were provided *ad libitum* to the dams from GD 10 until postnatal day (PND) 21 (weaning). After parturition, male and female offspring were identified by measuring the anogenital distance and genital papilla and/or nipple identification. The litter size was adjusted to eight pups/litter, and the gender ratio was 4:4 (female/male) as possible. Litters were maintained with the dams until the weaning period and weighed at PND 0, 6, 9, 12, 15, 18, 21 and 24. At PND 21, female offspring (1 pup/little) were allocated in their respective dietary intervention group (16 pups/group, 1 female/pup) and received the same diet as dams during gestation and lactation phases until PND 51 (puberty).

At PND 51, 10 female offspring/dietary group (1 female/litter) were euthanized, and abdominal mammary glands were removed for whole mount procedure. Other female offspring (n=16/group, 1 female/litter) received a single intragastric administration (ig) of DMBA (50 mg/kg, diluted in canole oil) [25,26] and was fed with adequate zinc diet until PND 180. All animals were examined three times per week to register the presence of gross mammary tumors, number and localization of each palpable mass in different mammary gland complexes. To ensure an adequate animal welfare, some animals were euthanized when a single tumor presented a mean diameter>2.5 cm or when the sum of mean diameter of multiple tumors reached the maximum burden of 2.5 cm [27]. The body weight was also registered individually once a week after DMBA administration. All animals were euthanized at PND 180 after fasted overnight, under sodium pentobarbital anesthesia (single ip dose of 30-mg/kg body weight). Immediately before euthanasia at PND 51, blood samples were collected by cardiac puncture to further determine serum zinc levels. Serum samples were mineralized in a 16-N nitric acid solution by microwave irradiation, and zinc level was determined from the mineralized materials by flameless AAS (GBC, Model 932 AA) [24].

2.3. Mammary gland whole mount staining and growth analyses

At PND 51, the right abdominal mammary glands from female offspring were removed. Briefly, mammary glands were air dried for 10 to 15 min on a clean glass slide and fixed in buffered formalin 10% for 48 h. The slides were washed in 70% ethanol, rinsed in water and stained with Carmine (1 g) and aluminum potassium sulfate dodecahydrate (2.5 g) (Sigma-Aldrich Co., EUA) for 2 days. Afterward, mammary whole mounts were dehydrated in sequential steps of ethanol (70%, 95% and 100%), cleared in xylene and mounted with Permount and coverslipped [28]. Mammary glands tree were photographed using magnifying glass at magnification 1× (Leica MZ12 – Japan - DF C 420) coupled to a capture system and image analysis. Three different parameters were measured for each mammary gland tree representing its outgrowth: ductal elongation, transverse growth and lateral growth [25,26,28]. The mean number of terminal end buds (TEBs) and terminal ducts (TDs) in external margin of mammary gland were determined [28] under a microscope Bx 53F (Olympus, Japan, 20× objective) in 10 offspring/group (1 female/litter).

2.4. Tissue processing and histology procedures

The mammary tumors were collected by incision of ventral pubic region until the chest. The number and localization of each palpable mass in the different mammary gland complexes were carefully recorded. The mammary tumor volume and area were measured macroscopically in three dimensions using a caliper rule, and the volume (cm^3) was calculated according to ellipsis volume formula: $4/3\pi \times (\text{length}/2) \times (\text{width}/2) \times (\text{depth}/2)$ [29], and stratified into three different categories: small (< 4.5 cm^3), medium (4.5 to 8.5 cm^3) or large (>8.5 cm^3). For histological analysis, tumor samples were collected and fixed in 10% phosphate-buffered formalin during 24 h, embedded in paraffin blocks, cut into 5-μm-thick sections and stained with hematoxylin/eosin (HE). Mammary lesions were classified according to previously published criteria [30]. Tumor samples were also snap-frozen in liquid nitrogen and stored at −80 C for gene expression analysis. Only papillary tumors were selected for RNA isolation and microarray analysis to exclude possible genomic expression heterogeneity among different tumor types.

2.5. RNA extraction and microarray procedure

Total RNA was isolated from mammary tumors using RNeasy Lipid Tissue Mini Kit (Qiagen Inc., Valencia, CA) following manufacture's protocol. After DNase I treatment, RNA concentration was measured using NanoVue spectrophotometer (GE Healthcare Life Sciences). RNA quality was assessed by capillary electrophoresis using Agilent 2100 Bioanalyzer (Agilent Technologies, Inc. Life Sciences and Chemical Analysis Group, Santa Clara, CA, USA).

Two hundred ng of total RNA from five tumors per group (each subject born from a different dam) were processed for microarray hybridization using 1-*Color Low Input Linear Amplification Kit* (Agilent) following the manufacturer's protocol. Labeled cRNAs were hybridized to microarray slides (Whole rat genome-4X44K oligo microarrays-G4131F, Agilent Technologies) at 65 °C for 17 h. Slides were then washed and scanned following Agilent's recommendations. The digitalized images were decoded by Agilent G4900DA Sure Scan Microarray Scanner System and submitted to quality control tests through Feature Extractor v.15.5 (Agilent Technologies, Inc. Life Sciences and Chemical Analysis Group, Santa Clara, CA, USA).

2.5.1. Microarray data analyses

Expression data were normalized and filtered, and statistical analysis was performed using the LIMMA package (Linear Models for Microarray Analysis) [31] on R platform. After importing the data generated by the Feature Extraction software, the arrays were normalized using the quantile method. Then nonspecific filters were

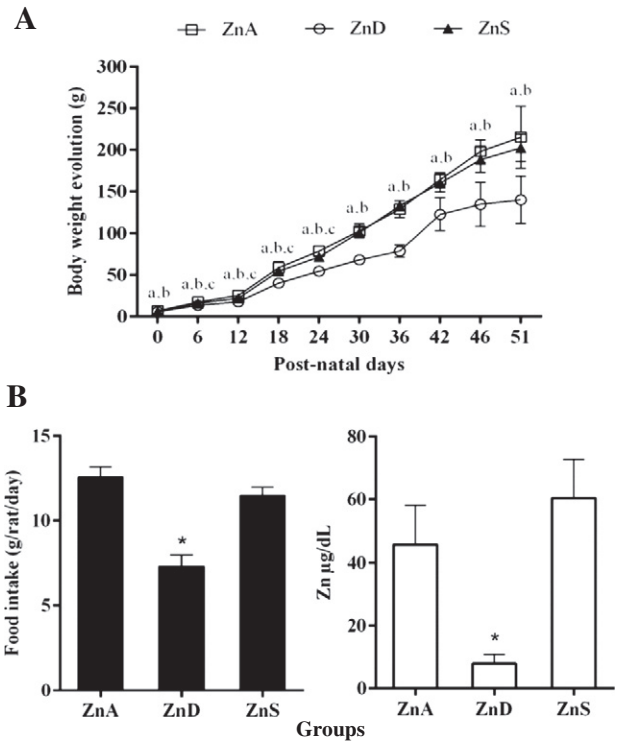

Fig. 1. Effects of diets with different dietary zinc levels on (A) body weight evolution from the birth (PND 0) until puberty (PND 51) and (B) food intake from weaning period (PND 21) until puberty (PND 51) in the different dietary zinc levels groups and serum zinc levels (µg/dL) at PND 51, ZnA: zinc-adequate diet (35-mg/kg chow), ZnD: zinc-deficient diet (3-mg/kg chow), ZnS: zinc-supplemented diet (180-mg/kg chow) intake during maternal and until puberty period (PND 51). Values expressed as mean ± S.E.M. Kruskal–Wallis test, with a Student–Newman–Keuls multiple comparison posttest. [a]ZnD < ZnA, [b]ZnD < ZnS, [c]ZnA > ZnS, $P<.05$; * Different from ZnA and ZnS groups, $P<.001$.

applied to ensure statistical analysis with only the high intensity probes: (a) it was calculated the 95% intensity value of the negative controls; 2) it was established, as a cutoff, the intensity value 10% above the previously calculated value; 3) it was selected, in all arrays, the probes that showed a higher value than the cutoff frequency in at least five samples of the experiment. In addition, there were selected only the probes with a valid Entrez ID. When selected probes had technical replicates within the same array, we used the replicates' average in the statistical analysis.

Statistical analysis was performed using Bayesian empirical model for differential expression (eBayes) set up in LIMMA package, using as weights the quality of each array. Finally, there were considered statistically significant genes with False Discovery Rate value (FDR) of less than 0.15, P value less than 0.05 and fold change greater than 1.5.

The statistically significant genes were subjected to analysis of gene ontology (GO) using GOstats package and rgug4131a.db platform R. The GO terms were considered statistically significant when P value was less than 0.01. The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus through GEO Series accession number GSE86270 [32].

2.5.2. Validation by real-time PCR

In order to validate the expression of selected genes found in the microarray analysis, real-time PCR was performed on the same RNA samples used for microarray. Complementary DNA was synthesized using de High capacity Kit (Applied Biosystems, USA) according to the manufacturer instructions. Total RNA was reverse transcribed using 6 µL of random hexamer primer (10×), 6 µL of reaction buffer (10×), 2.5 µL of dNTPs (25×) and 3 µL of Multiscribe enzyme (50 U/µL). This mixture was incubated at 25 °C for 10 min and then at 37 °C for 2 h, and it was kept at 4 °C. Subsequently, each cDNA was stored at −20 °C. TaqMan/FAM-MGB probes and primers for *A2m* (Rn00560589_m1), *Bcl3* (Rn00578225_m1) and *Cxcl1* (Rn01457652_m1) were used for amplification. Reactions were performed at 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. β-actin was used as endogenous control. Taqman Universal PCR Master Mix was also obtained from Applied Biosystems, and quantitative real-time PCR was performed in duplicate in a 7500 FAST PCR system (Applied Biosystems, USA). Relative gene expression data were analyzed using the 2-ΔΔCT method [33].

2.6. Statistical analysis

Body weight evolution and food intake, as well as mammary gland outgrowth, number of TEBs and TDs, tumor latency and multiplicity were analyzed by ANOVA or Kruskal–Wallis tests. In addition, mammary gland outgrowth was further analyzed by ANCOVA test, using body weight as covariable. The statistical analysis of tumor size, incidence and different histological tumor types were examined using chi-square test or the Fischer' Exact Test. The tumor-free survival was analyzed by Cox proportional-hazards analysis model. Statistical analysis was performed using the Jandel Sigma Stat Software (Jandel Corporation, San Rafael, CA, USA), and generation of graphs was performed using GraphPad Prism software (Version 6.01, La Jolla, CA, USA). Statistical differences were considered significant with $P<.05$.

3. Results

3.1. General observations

At birth (PND 0), body weight from female offspring from dams receiving ZnD was significantly lower ($P=.018$) in comparison to

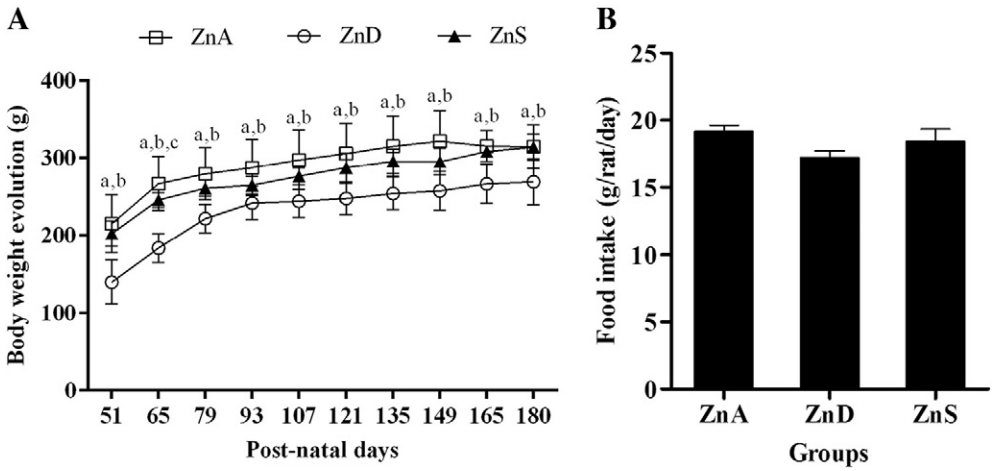

Fig. 2. Effects of exposure to diets with different dietary zinc levels on (A) body weight evolution and (B) food intake from PND 51 (DMBA administration) PND 180 in the different dietary zinc levels groups, ZnA: zinc-adequate diet (35-mg/kg chow), ZnD: zinc-deficient diet (3-mg/kg chow), ZnS: zinc-supplemented diet (180-mg/kg chow) intake during maternal and until puberty period (PND 51). All animals turned to ZA diet during PND 51–180. Values expressed as mean ± S.E.M. Kruskal–Wallis test, with a Dunn's multiple comparison posttest. [a]ZnD < ZnA, [b]ZnD < ZnS, [c]ZnA > ZnS, $P<.05$.

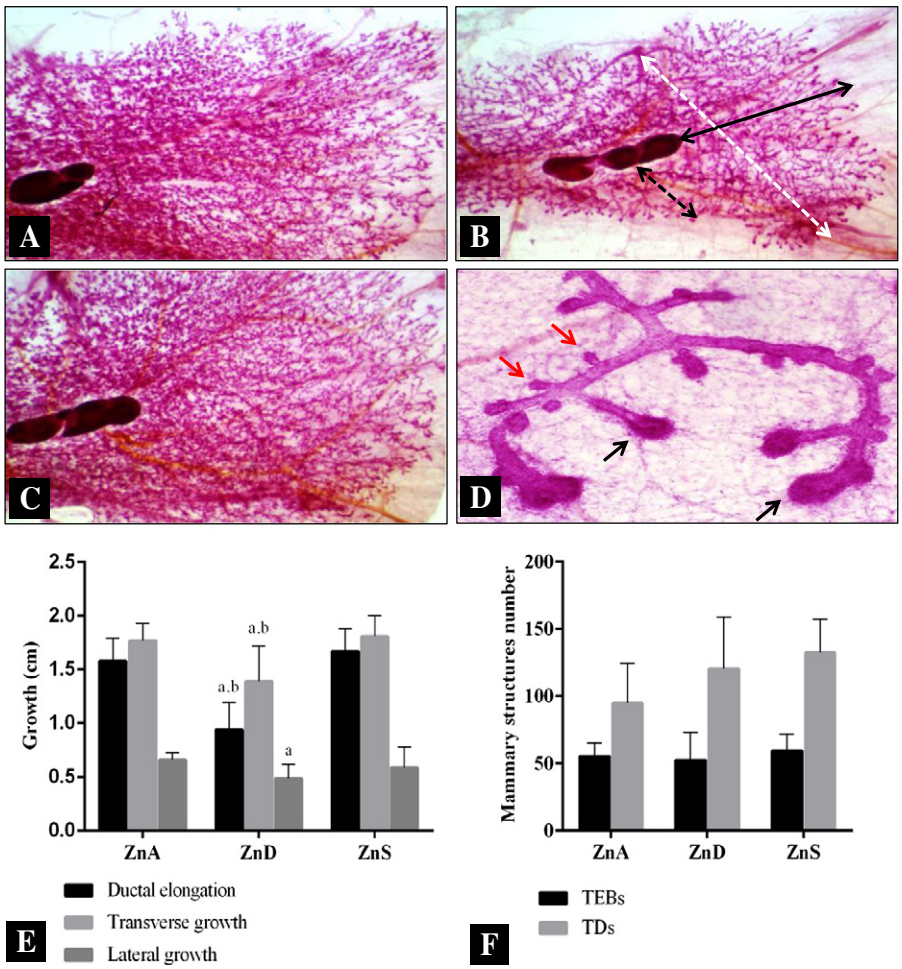

Fig. 3. Mammary gland whole mount of female offspring exposed to diets with different dietary zinc levels during maternal until puberty period (PND 51). (A) Mammary gland from ZnA (zinc-adequate diet - 35-mg/kg chow). (B) Mammary gland from ZnD (zinc-deficient diet - 3-mg/kg chow) group, showing examples of criteria to evaluate mammary gland outgrowth, such as ductal elongation (black arrow), transverse growth (white dotted arrow) and lateral growth (black dotted arrow). (C) Mammary gland from ZnS (zinc-supplemented diet - 180-mg/kg chow) in magnifying glass at magnification ×1. (D) Terminal end buds (TEBs) (black arrows) and terminal ducts (TDs) (red arrows) in microscope at magnification ×20. (E) Effects of intake of dietary zinc status on mammary gland outgrowth. (F) Mean number of TEBs and TDs in different groups. Values expressed as mean ± S.E.M. ANOVA test, with a Holm-sidak multiple comparison posttest. [a]ZnD < ZnA, [b]ZnD < ZnS, $P<.05$.

those that received maternal ZnA and ZnS during fetal life (Fig. 1A). This difference in body weight remained from DPN 6 to 51 ($P=.001$) (Fig. 1A). From PND 6 to 24, there was a significant reduction ($P=.001$) in body weight from female offspring receiving ZnS in comparison to control (ZnA) group (Fig. 1A).

During prepubertal and early pubertal periods (PND 21 to 51), a significant reduction in food consumption was observed in female offspring receiving ZnD when compared to the other groups ($P=.001$) (Fig. 1B). At PND 51, serum zinc levels in female offspring were significantly influenced by the low zinc intake in comparison to ZnA group ($P<.001$) but not significantly by the zinc supplementation; altogether it was observed an increase in ZnS group in relation to the ZnA group (Fig. 1B). Even after receiving ZnA (after DMBA administration), ZnD group continued showing significantly lower body weight throughout the experimental period (PND 51 to 180) ($P=.001$) when compared to the other groups (Fig. 2A). In this period, the food consumption did not differ among the groups (Fig. 2B).

3.2. Mammary gland development

Representative images from the mammary glands of each dietary group, TEBs and TDs are illustrated in Fig. 3A–D. Mammary gland outgrowth parameters and mean number of TEBs and TDs evaluated at PND 51 are presented in Fig. 3D and E. No differences were found in the mean number of TEBs and TDs among groups (Fig. 3F). There was found a significant reduction of ductal elongation and transverse

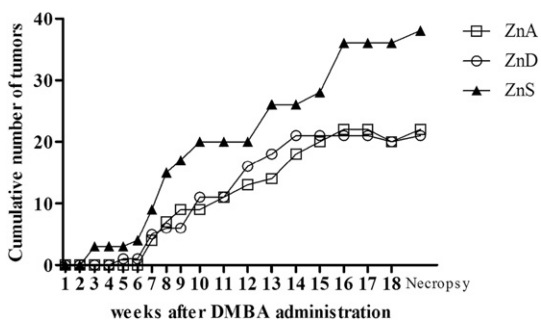

Fig. 4. The cumulative number of palpable mammary tumors as a function of time after 7,12-dimethylbenz(a)anthracene (DMBA) administration and more those identified at necropsy is showed (all histologically verified). ZnA: zinc-adequate diet (35-mg/kg chow), ZnD: zinc-deficient diet (3-mg/kg chow), ZnS: zinc-supplemented diet (180-mg/kg chow) intake during maternal until puberty period (PND 51).

Table 1
Latency, incidence, multiplicity and histological types of DMBA-induced mammary tumors in the different dietary zinc levels groups

Parameters	Groups[2]		
	ZnA	ZnD	ZnS
Number of animals	16	16	16
Tumor latency (days)[1]	78.38±22.18	70.89±19.08	72.90±24.66
First tumor (day)	45°	36°	20°
Tumor incidence (%)[3]	11/16 (69)	11/16 (69)	14/16 (81)
Multiplicity[1, 4]	2.00±1.10	1.91±1.38	2.85±1.99
Number of tumors	22	21	38
Tumor size (number and %)			
Small	16 (72)	11 (52)	24 (63)
Medium	4 (18)	7 (33)	9 (24)
Large	2 (9)	3 (14)	5 (13)
Histological types (%)			
Papillary adenocarcinoma	17 (77)	16 (76)	21 (55)
Tubular adenocarcinoma	3 (14)	4 (19)	11 (29)
Tubular/Papillar adenocarcinoma	2 (9)	0 (0)	3 (8)
Cribiform adenocarcinoma	0 (0)	1 (5)	3 (8)

[1] Values are mean ± SD.
[2] ZnA: Zinc-adequate diet (35-mg/kg chow), ZnD: zinc-deficient diet (3-mg/kg chow) and ZnS: zinc-supplemented diet (180-mg/kg chow) intake during maternal until puberty period (PND 51).
[3] Palpable tumors (and histologically verified) and tumor identified after necropsy.
[4] Number of tumors per animal; small tumor (< 4.5 cm^3), medium (4.5 to 8.5 cm^3) and large (>8.5 cm^3).

growth (P=.001 and P=.002, respectively) of mammary gland in ZnD-fed offspring when compared to other groups. In addition, there was a significant reduction (P=.045) of mammary gland lateral growth in ZnD-fed offspring in comparison to control (ZnA) group. In order to analyze whether these differences were directly correlated to dietary Zn status or body weight, we performed analysis of covariance (ANCOVA test), using body weight as covariable. There was no significant effect of treatment on ductal elongation (P=.054) and lateral growth (P=.089) after controlling for body weight as covariable. Body weight, in turn, was significantly related to ductal elongation (P=.000002) and lateral growth (P=.000189).

3.3. Mammary tumors and histopathological analyses

Fig. 4 represents the cumulative number of palpable mammary tumors detected throughout the experimental period (1 to 18 weeks after DMBA administration) as well as the tumors identified at necropsy. Female offspring from ZnS group developed the first palpable tumor at 3th week after carcinogen administration, whereas ZnA and ZnD groups developed at 7th and 5th weeks after, respectively (Fig. 4). At the end of the experiment, the female offspring that received a previous zinc-supplemented diet developed 72% more mammary tumors than those that received zinc-adequate diet (38 tumors *vs.* 22 tumors, respectively, P<.05). Moreover, female offspring provided with previous ZnD developed a similar number of mammary tumors (21 tumors) in relation to control (ZnA) group (Table 1). There were no significant differences in mammary tumor incidence, latency or multiplicity as well as the tumor volume noticed among the dietary groups (Table 1). However, the ZnS group presented lower percentage of tumor-free animals, indicating a tendency of developing higher mammary tumor incidence (Fig. 5).

The histopathological analyses showed that most of the DMBA-induced mammary tumors were invasive adenocarcinomas presenting predominantly papillary pattern (Fig. 6A) in 77%, 76% and 55% of animals for the zinc-deficient, adequate and supplemented diets (ZnA, ZnD and ZnS) groups, respectively (Table 1).

3.4. Microarray expression and qRT-PCR validation

As shown in Table 2, from about 40,000 probes, 34 genes were identified as differentially expressed in the tumors derived from ZnD and ZnS groups when compared to control (ZnA) group. In the tumors derived from ZnD group, 9 genes were down-regulated, and 6 genes were up-regulated. Gene ontology enrichment analysis revealed that, overall, these genes were involved in regulation of DNA-dependent transcription, transport of oxygen, regulation of cyclin-dependent kinases (CDKs) and cell adhesion (Supplementary Table 1). The genes present in these enriched GOs were *Cdkn1a*, *Cyba*, *Penk* and *Rasd1*. In the tumors derived from ZnS group, 15 genes were down-regulated, and 4 genes were up-regulated. These genes are involved in cell adhesion, organelles and histones organization, lectin pathway complement regulation, chemotaxis and metabolic process of coenzymes (Supplementary Table 1). The genes present in these enriched GOs were *A2m*, *Cxcl1*, *Fmod*, *H1f0*, *Hist1h2af* and *Hist3h2a*.

Due to the biological function of the altered genes and their potential relevance on mammary carcinogenesis, *A2m*, *Cxcl1* and *Bcl3* (*LOC680611*) genes were chosen for further qRT-PCR evaluation. As shown in Fig. 7, real-time PCR data showed significant up-regulation of *Bcl3* and *Cxcl1* genes (P=.016 and 0.038, respectively) in ZnS group when compared to control (ZnA) group. In addition, the *A2m* gene was found to be up-regulated in this comparison, but no statistically significant difference was found.

4. Discussion

The findings of the present study showed that early-in-life zinc deficiency did not alter the susceptibility to chemically induced mammary carcinogenesis in adulthood. On the other hand, zinc supplementation increased the total number of mammary tumors (~72%) accompanied by a distinct gene expression pattern in comparison to control (ZnA) group, indicating a potential increase in the susceptibility to DMBA-induced mammary tumors.

We found that zinc restriction during prenatal and postnatal phases induced a significant reduction in body weight evolution and food intake in female offspring from ZnD group, corroborating with the findings of previous described studies [22,34]. This finding may be explained by studies that observed hypogeusia (decreased gustatory sense) in both humans and rats induced by zinc deficiency, leading ultimately in reduction of food consumption [35,36].

Some studies have shown that low zinc intake (3-mg/kg chow) during adulthood can suppress mammary tumorigenesis in female SD rats [16,17]. Other studies have demonstrated that marginal zinc-deficient diet (15-mg Zn/kg chow) during postinvolution mammary gland phase resulted in high zinc accumulation in mammary gland of

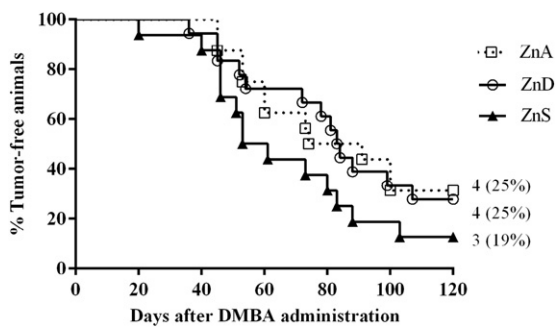

Fig. 5. Percentage of palpable tumor-free female rats from ZnA (zinc-adequate diet - 35-mg/kg chow), ZnD (zinc-deficient diet—3-mg/kg chow) and ZnS (zinc-supplemented diet - 180-mg/kg chow) groups (n=16 per group). No significant difference (P>.05) according to Cox proportional-hazards analysis model.

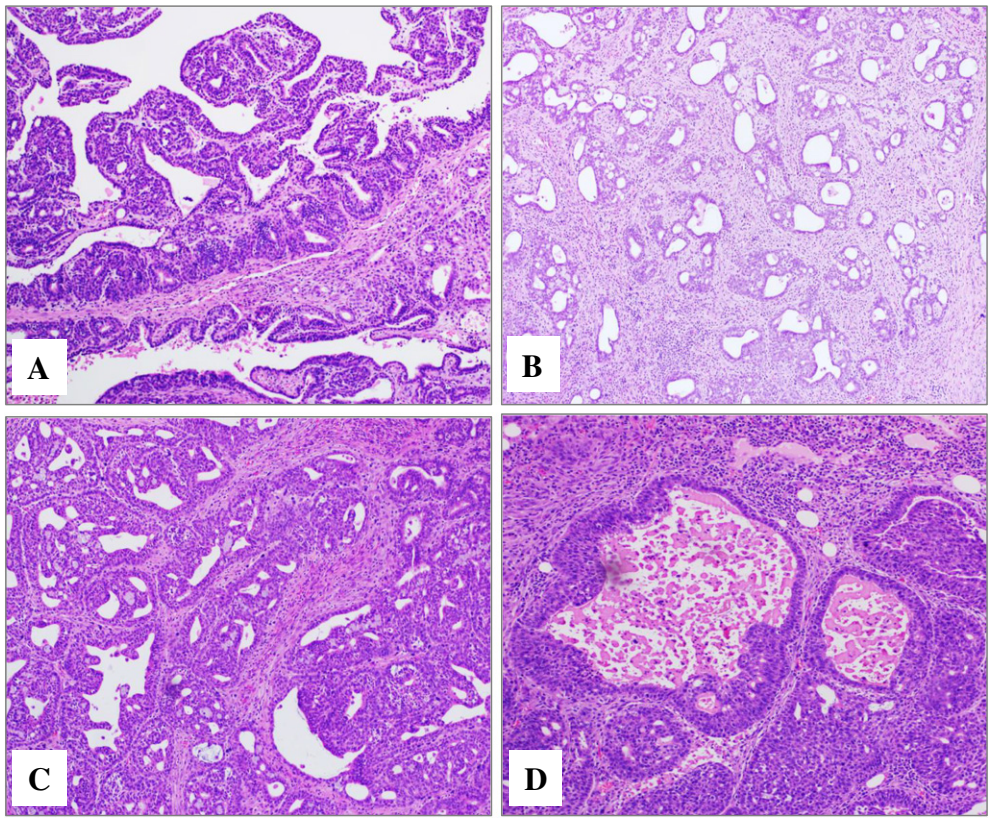

Fig. 6. Representative images of HE-stained mammary tumor sections at PND 180: (A) Papillary adenocarcinoma; (B) tubular adenocarcinoma; (C) Cribiform adenocarcinoma; (D) Cribiforme/Comedo adenocarcinoma, 10×.

adult female C57/Bl6 mice, leading to shorter tumor latency, and greater incidence of tumors in these animals, when DMBA was administered to dams [20,37]. Although some studies have indicated that nutritionally induced poor early growth is linked to increased susceptibility of mammary tumor development in female Wistar rats [38,39], the altered body weight and mammary gland outgrowth induced by dietary zinc deficiency did not alter the development of chemically induced mammary tumors in our study.

The findings indicating a potential increase in DMBA-induced mammary tumors susceptibility in ZnS group are interesting. Previous studies have suggested that elevated zinc levels in cancerous tissue may be associated to increased risk of human breast cancer development [40]. In addition, other studies suggested that the zinc is essential for tumor growth and mammary tumorigenesis [6,16,17]. These findings may be explained by the evidence that the intracellular increase of zinc is essential for cell proliferation, since its absence is related to tumor cells arrest in S-phase [41,42]. On the other hand, excessive intracellular zinc accumulation is cytotoxic and presents a potential prooxidant activity leading to cellular oxidative stress and increased risk of mammary cancer development in mice [20,37,43]. In the present study, the dietary zinc supplementation (~fivefold more than the control) could have been cytotoxic to the mammary tissue, corroborating to other studies [44,45] and modifying carcinogenic action of DMBA on this tissue leading to higher number of mammary tumors in this group. *In vitro* studies have shown that the tissue zinc excess increases production of reactive oxygen species (ROS), inflammation and intracellular acidosis [46–48], contributing to genomic instability and risk of mammary tumor development [20,49]. Thus, increased intracellular ROS levels, taking to the oxidative DNA damage [49], could be contributing significantly to increase the deleterious effects of DMBA on the mammary gland.

To elucidate whether previous dietary zinc status modifies tumor gene expression signature, global gene expression analysis was performed in papillary mammary tumors of all groups. There were 15 and 19 genes whose expression was altered in the tumors from ZnD and ZnS groups, respectively, in comparison to the control (ZnA) group. From the differentially expressed genes, we chose and validated by qPCR-RT the up-regulation of *Bcl3* and *Cxcl1* genes in tumors from ZnS group.

Bcl3 is a proto-oncogene from *IkB* family that has been detected in several types of cancer, including breast cancer [50–52]. Studies show that *Bcl3* overexpression could confer an adaptive advantage of the tumor cells [53,54], since this gene may be related to antiapoptotic function, through dimerization with *p52* and subsequent transactivation of *Bcl2* [55]. This adaptive advantage could also be granted through increase of cell proliferation and survival [56,57] induced by the expression of cyclin D1, leading to an increased G1/S cell cycle phase [58]. Moreover, it has been described that this oncogene is up-regulated by several cytokines, such as tumor necrosis factor alpha (*TNF alpha*) [59,60], IL-4 [61], IL1 [62,63], IL6 [64], IL-10 [65] and IL-12 [66]. Besides, it is a cofactor of the inflammatory mediator, factor nuclear kappa B (NF-kB) [67]. In this regard, it was also found that the *Cxcl1* gene is up-regulated in tumor samples of zinc-supplemented diet (ZnS) group. *Cxcl1* is an inflammatory chemokine and potent angiogenic/lymphangiogenic growth factor, which could contribute to cancer progression and chemoresistance [68–70]. In addition, this gene promotes the migration of breast cancer cells *in vitro* [71] and tumor growth and metastasis, as well as angiogenesis in mouse squamous cell carcinoma [72]. Therefore, the elevated expression of *Bcl3* and *Cxcl1* genes may contribute, in part, to the trend of worse outcome in ZnS compared to ZnA group, demonstrated by lower percentage of

Table 2
Up- and down-regulated genes from ZnD and ZnS groups compared to control group

Probe ID	Gene symbol	Gene name	Fold change
(a) ZnD *vs.* ZnA			
A_42_P749184	Penk-rs	preproenkephalin, related sequence	−2.8153062
A_42_P843394	Pcolce	procollagen C-endopeptidase enhancer protein	−2.1212477
A_44_P245835	RGD1310110_predicted	similar to 3632451O06Rik protein (predicted)	−1.9286294
A_44_P463749	Rasd1	RAS, dexamethasone-induced 1	−1.8845004
A_44_P161846	Hist3h2ba_predicted	histone 3, H2ba (predicted)	−1.643933
A_44_P914438	Cdkn1a	cyclin-dependent kinase inhibitor 1A	−1.5824675
A_42_P541032	Ftl1	ferritin light chain 1	−1.5824384
A_44_P929423	LOC687154	similar to integrin alpha 9	−1.564092
A_43_P12413	Cyba	cytochrome b-245, alpha polypeptide	−1.5084457
A_42_P525454	Tenc1_predicted	tensin like C1 domain containing phosphatase (predicted)	1.53401065
A_43_P23403	Dsc3_predicted	desmocollin 3 (predicted)	1.54010703
A_43_P10089	Slc45a3_predicted	solute carrier family 45, member 3 (predicted)	1.64104682
A_44_P335402	Mocos_predicted	molybdenum cofactor sulfurase (predicted)	1.70695417
A_44_P326147	LOC307495	similar to biliverdin reductase B (flavin reductase (NADPH)	1.98810006
A_44_P139956	MGC72973	beta-glo	2.36119931
(b) ZnS *vs.* ZnA			
A_42_P670631	RGD1305645_predicted	similar to RIKEN cDNA 1500015O10 (predicted)	−3.0704894
A_43_P15590	Fmod	fibromodulin	−2.3217546
A_44_P339798	LOC680522	similar to Histone H1.5 (H1 VAR.5) (H1b)	−2.0120397
A_44_P136730	LOC682543	similar to histone 2a	−1.977306
A_44_P1059985	Spbc24_predicted	spindle pole body component 24 homolog (*S. cerevisiae*) (predicted)	−1.9057844
A_44_P245835	RGD1310110_predicted	similar to 3632451O06Rik protein (predicted)	−1.8714904
A_44_P1052067	Plekha4	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 4	−1.7899195
A_42_P611911	Car15_predicted	carbonic anhydrase 15 (predicted)	−1.7589822
A_44_P396522	H2a	histone 2a	−1.6633457
A_44_P527154	RGD1564767_predicted	Histone H2a (predicted)	−1.6364251
A_44_P212835	RGD1562827_predicted	similar to Histone H2A.1 (predicted)	−1.6139213
A_44_P433431	Hist1h2ao_predicted	histone 1, H2ao (predicted)	−1.5888431
A_44_P172950	Hist1h2an_predicted	histone 1, H2an (predicted)	−1.5744371
A_44_P210563	H1f0	H1 histone family, member 0	−1.5400996
A_44_P222551	Hist2h2aa_predicted	histone 2, H2aa (predicted)	−1.5314121
A_43_P14162	A2m	alpha-2-macroglobulin	1.50187406
A_44_P335402	Mocos_predicted	molybdenum cofactor sulfurase (predicted)	1.51025951
A_42_P577431	LOC680611 (Bcl3)	similar to B-cell leukemia/lymphoma 3	1.57715025
A_42_P473398	Cxcl1	chemokine (C-X-C motif) ligand 1	1.75318841

tumor-free animals, higher cumulative number of tumors and an earlier tumor development.

Some authors suggest that the biological effects of zinc can be carcinogenic or protective, and it seems to be very complex and manifest in cancer-dependent manner [49]. Therefore, there is an ambivalent effect of zinc supplementation. In recommended daily dosage 15 mg/day, zinc may inhibit tumorigenesis, while in 10-fold higher dosage (higher than 150 mg/day), zinc may promote it [49,72]. Proper cellular zinc status profoundly affects cellular health and has numerous roles in cancer initiation, progression and, potentially, in cancer prevention [2,12,16]. Although a correlation between increased TEB number and breast cancer susceptibility has been described in maternal and early-in-life intervention studies [28], the potential increase in the susceptibility to DMBA-induced mammary tumors by zinc supplementation was not associated with alterations in mammary gland development in the female offspring.

The intestine is the site of zinc absorption, and the major route of zinc excretion, since the enterocytes express some zinc transporters (ZnT) and zinc importers (Zip), including ZnT1, ZnT2, ZnT4, ZnT5, ZnT6, ZnT7, Zip4 and Zip5 [73]. In a previous study, using a similar dietary zinc status, male SD rats fed a low zinc diet (<1-mg Zn/kg) for 2 weeks presented a reduction in ZnT-2 mRNA in small intestine and kidney when compared with an adequate zinc intake (30-mg Zn/kg). On the other hand, ZnT-1 and ZnT-2 mRNAs were significantly higher in both tissues when a zinc supplementation intake (180-mg Zn/kg) was provided. These data provide evidence that zinc transporter expression is responsive to dietary zinc status under physiologically relevant conditions [74], while systemic homeostasis and efficient regulatory mechanisms on the cellular level generally prevent the uptake of cytotoxic doses [44,45]. Chung and collaborators showed that patients receiving ZnD diet containing 0.6-mg Zn/day for 1 week and 4-mg Zn/day for 5 weeks presented plasma Zn increasing after returning to adequate zinc diet containing 11-mg Zn/day for 4 weeks with 20-mg supplemental Zn/day for first 7 days [75]. Therefore, further experiments are warranted to investigate serum zinc levels and specific ZnT and Zip genes expression in small intestine after a short-term of supply of zinc-adequate diet for ZnD and ZnS groups for a better interpretation of modifying effects of dietary zinc status on DMBA-induced tumor initiation.

In conclusion, we found that early-in-life zinc deficiency lead to reduced food consumption and lower body weight evolution in adulthood female offspring, possibly due to a nutritional reprogramming caused by dietary zinc deficiency. On the other hand, the present study provided evidence that early-in-life zinc supplementation signals to increased susceptibility to DMBA-induced mammary tumor development possibly by changes in gene expression of proto-oncogenes and cytokines. However, further evaluation of potential role of zinc and its molecular mechanisms involved in mammary tumorigenesis are required.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2017.03.003.

Acknowledgements

Flávia R.M. da Silva and Joyce R. Zapaterin were recipient of a fellowship from CAPES and FAPESP (2013/15060-9), respectively. Luis F. Barbisan was recipient of support research from FAPESP (2012/13004-1).

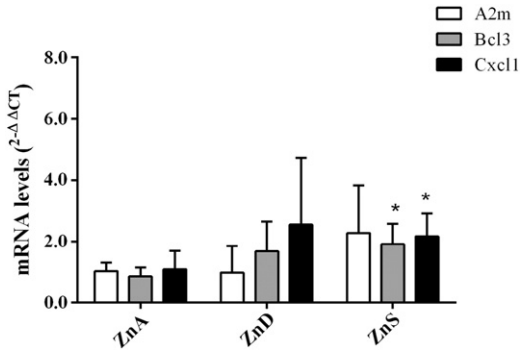

Fig. 7. Real-time PCR validation of genes found in the microarray analysis of mammary tumor samples according to treatments: ZnA (zinc-adequate diet - 35-mg/kg chow), ZnD (zinc-deficient diet−3-mg/kg chow) and ZnS (zinc-supplemented diet - 180-mg/kg chow). Values are mean ± S.D. * Different from the control group (ZnA) when *P* value<.05.

References

[1] Chasapis CT, Loutsidou AC, Spiliopoulou CA, Stefanidou ME. Zinc and human health: an update. Arch Toxicol 2012;86:521–34. http://dx.doi.org/10.1007/s00204-011-0775-1.

[2] Prasad AS, Beck FWJ, Snell DC, Kucuk O. Zinc in cancer prevention. Nutr Cancer 2009;61:879–87. http://dx.doi.org/10.1080/01635580903285122.

[3] Sharif R, Thomas P, Zalewski P, Fenech M. The role of zinc in genomic stability. Mutat Res 2012;733:111–21. http://dx.doi.org/10.1016/j.mrfmmm.2011.08.009.

[4] Lim KH, Riddell LJ, Nowson CA, Booth AO, Szymlek-Gay EA. Iron and zinc nutrition in the economically-developed world: a review. Forum Nutr 2013;5(8):3184–211. http://dx.doi.org/10.3390/nu5083184.

[5] Stathopoulou MG, Kanoni S, Papanikolaou G, Antonopoulou S, Nomikos T, Dedoussis G. Mineral intake. Prog Mol Biol Transl Sci 2012;108:201–36. http://dx.doi.org/10.1016/B978-0-12-398397-8.00009-5.

[6] Alam S, Kelleher SL. Cellular mechanisms of zinc dysregulation: a perspective on zinc homeostasis as an etiological factor in the development and progression of breast cancer. Nutrients 2012;4:875–903. http://dx.doi.org/10.3390/nu4080875. Mao S, Huang S. Zinc and copper levels in bladder cancer: a systematic review and meta-analysis. Biol Trace Elem Res 2012;153:5–10. http://dx.doi.org/10.1007/s12011-013-9682-z.

[7] Al-Ebraheem A, Farquharson MJ, Ryan E. The evaluation of biologically important trace metals in liver, kidney and breast tissue. Appl Radiat Isot 2009;67:470–4. http://dx.doi.org/10.1016/j.apradiso.2008.06.018.

[8] Johnson LA, Kanak MA, Kajdacsy-Balla A, Pestaner JP, Bagasra O. Differential zinc accumulation and expression of human zinc transporter 1 (hZIP1) in prostate glands. Methods 2010;52:316–21. http://dx.doi.org/10.1016/j.ymeth.2010.08.004.

[9] Cortesi M, Fridman E, Volkov A, Shilstein SS, Chechik R, Breskin A, et al. Clinical assessment of the cancer diagnostic value of prostatic zinc: a comprehensive needle-biopsy study. Prostate 2008;68:994–1006. http://dx.doi.org/10.1002/pros.20766.

[10] da Silva FRM, Dias MC, Barbisan LF, Rodrigues MAM. Lack of protective effects of zinc gluconate against rat colon carcinogenesis. Nutr Cancer 2013;65:571–7. http://dx.doi.org/10.1080/01635581.2013.775317.

[11] Ban KA, Godellas CV. Epidemiology of breast cancer. Surg Oncol Clin N Am 2014;23:409–22. http://dx.doi.org/10.1016/j.soc.2014.03.011.

[12] Zapaterini JR, Silva FRM, Maxwell M, Barbisan LF. The role of zinc in carcinogenesis. In: Morton R, editor. Zinc deficiency: Etiology, screening, methods and health implications. 1st ed. New York: Nova Science Publisher; 2016. p. 39–80 [Cap. 3].

[13] Tinoco-Veras CM, Bezerra Sousa MS, da Silva BB, Franciscato Cozzolino SM, Viana Pires L, Coelho Pimentel JA, et al. Analysis of plasma and erythrocyte zinc levels in premenopausal women with breast cancer. Nutr Hosp 2011;26:293–7. http://dx.doi.org/10.1590/S0212-16112011000200008.

[14] Taylor K, Gee J, Kille P. Zinc and cancer. In: Rink L, editor. Zinc in human health. IOS Press Aachen Germany; 2011.

[15] Borges de Araújo CG, Oliveira do Nascimento Holanda A, de Souza Rocha CV, Soares do Nascimento AP, Simplício Revoredo CM, Borges da Silva B, et al. Relationship between zincemia, superoxide dismutase activity and marker of oxidative stress in women with breast cancer. Nutr Hosp 2015;32:785–91. http://dx.doi.org/10.3305/nh.2015.32.2.9204.

[16] Lee S, Simpson M, Nimmo M, Xu Z. Low zinc intake suppressed N-methyl-N-nitrosourea-induced mammary tumorigenesis in Sprague-Dawley rats. Carcinogenesis 2004;25:1879–85. http://dx.doi.org/10.1093/carcin/bgh214.

[17] Lee R, Woo W, Wu B, Kummer A, Duminy H, Xu Z. Zinc accumulation in N-methyl-N-nitrosourea-induced rat mammary tumors is accompanied by an altered expression of ZnT-1 and metallothionein. Exp Biol Med (Maywood) 2003;228:689–96.

[18] Eide DJ. The oxidative stress of zinc deficiency. Metallomics 2011;3:1124–9. http://dx.doi.org/10.1039/c1mt00064k.

[19] Hashemi M, Ghavami S, Eshraghi M, Booy EP, Los M. Cytotoxic effects of intra and extracellular zinc chelation on human breast cancer cells. Eur J Pharmacol 2007;557:9–19. http://dx.doi.org/10.1016/j.ejphar.2006.11.010.

[20] Bostanci Z, Mack RP, Lee S, Soybel DI, Kelleher SL. Paradoxical zinc toxicity and oxidative stress in the mammary gland during marginal dietary zinc deficiency. Reprod Toxicol 2015;54:84–92. http://dx.doi.org/10.1016/j.reprotox.2014.07.076.

[21] Tomat AL, Inserra F, Veiras L, Vallone MC, Balaszczuk AM, Costa MA, et al. Moderate zinc restriction during fetal and postnatal growth of rats: effects on adult arterial blood pressure and kidney. Am J Physiol Regul Integr Comp Physiol 2008;295:R543–9. http://dx.doi.org/10.1152/ajpregu.00050.2008.

[22] Tomat A, Elesgaray R, Zago V, Fasoli H, Fellet A, Balaszczuk AM, et al. Exposure to zinc deficiency in fetal and postnatal life determines nitric oxide system activity and arterial blood pressure levels in adult rats. Br J Nutr 2010;104:382–9. http://dx.doi.org/10.1017/S0007114510000759.

[23] Tomat AL, Juriol LV, Gobetto MN, Veiras LC, Mendes Garrido Abregú F, Zilberman J, et al. Morphological and functional effects on cardiac tissue induced by moderate zinc deficiency during prenatal and postnatal life in male and female rats. Am J Physiol Heart Circ Physiol 2013;305:H1574–83. http://dx.doi.org/10.1152/ajpheart.00578.2013.

[24] Romualdo GR, Goto RL, Henrique Fernandes AA, Cogliati B, Barbisan LF. Dietary zinc deficiency predisposes mice to the development of preneoplastic lesions in chemically-induced hepatocarcinogenesis. Food Chem Toxicol 2016;96:280–9. http://dx.doi.org/10.1016/j.fct.2016.08.020.

[25] de Oliveira Andrade F, Fontelles CC, Rosim MP, de Oliveira TF, de Melo Loureiro AP, Mancini-Filho J, et al. Exposure to lard-based high-fat diet during fetal and lactation periods modifies breast cancer susceptibility in adulthood in rats. J Nutr Biochem 2014 Jun;25(6):613–22. http://dx.doi.org/10.1016/j.jnutbio.2014.02.002.

[26] Guido LN, Fontelles CC, Rosim MP, Pires VC, Cozzolino SM, Castro IA, et al. Paternal selenium deficiency but not supplementation during preconception alters mammary gland development and 7,12-dimethylbenz[a]anthracene-induced mammary carcinogenesis in female rat offspring. Int J Cancer 2016;139(8):1873–82. http://dx.doi.org/10.1002/ijc.30223.

[27] Workman P, Aboagye EO, Balkwill F, Balmain A, Bruder G, Chaplin DJ, et al. Guidelines for the welfare and use of animals in cancer research. Br J Cancer 2010;102(11):1555–77. http://dx.doi.org/10.1038/sj.bjc.6605642.

[28] de Assis S, Warri A, Cruz MI, Hilakivi-Clarke L. Changes in mammary gland morphology and breast cancer risk in rats. J Vis Exp 2010. http://dx.doi.org/10.3791/2260.

[29] Chatterjee M, Janarthan M, Manivannan R, Rana A, Chatterjee M. Combinatorial effect of fish oil (Maxepa) and 1alpha,25-dihydroxyvitamin D(3) in the chemoprevention of DMBA-induced mammary carcinogenesis in rats. Chem Biol Interact 2010;188:102–10. http://dx.doi.org/10.1016/j.cbi.2010.06.007.

[30] Russo J, Russo IH. Atlas and histologic classification of tumors of the rat mammary gland. J Mammary Gland Biol Neoplasia 2000;5:187–200.

[31] Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004;3. http://dx.doi.org/10.2202/1544–6115.1027 [Article 3].

[32] Edgar R, Domrachev M, Lash AE. Gene expression omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 2002;30:207–10.

[33] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 2001;25:402–8. http://dx.doi.org/10.1006/meth.2001.1262.

[34] Yu X, Jin L, Zhang X, Yu X. Effects of maternal mild zinc deficiency and zinc supplementation in offspring on spatial memory and hippocampal neuronal ultrastructural changes. Nutrition 2013;29:457–61. http://dx.doi.org/10.1016/j.nut.2012.09.002.

[35] Goto T, Komai M, Suzuki H, Furukawa Y. Long-term zinc deficiency decreases taste sensitivity in rats. J Nutr 2001;131:305–10.

[36] Hamano H, Yoshinaga K, Tanaka T, Eta R, Horii T, Kawabata Y, et al. Polaprezinc, a zinc compound, is distributed to the lingual epithelium and increases its zinc concentration in zinc-deficient rats. Life Sci 2009;85:759–64. http://dx.doi.org/10.1016/j.lfs.2009.10.006.

[37] Bostanci Z, Mack RP, Enomoto LM, Alam S, Brown A, Neumann C, et al. Marginal zinc intake reduces the protective effect of lactation on mammary gland carcinogenesis in a DMBA-induced tumor model in mice. Oncol Rep 2016;35:1409–16. http://dx.doi.org/10.3892/or.2015.4508.

[38] Fernandez-Twinn DS, Ekizoglou S, Gusterson BA, Luan J, Ozanne SE. Compensatory mammary growth following protein restriction during pregnancy and lactation increases early-onset mammary tumor incidence in rats. Carcinogenesis 2007;28:545–52. http://dx.doi.org/10.1093/carcin/bgl166.

[39] Fernandez-Twinn DS, Ekizoglou S, Martin-Gronert MS, Tarry-Adkins J, Wayman AP, Warner MJ, et al. Poor early growth and excessive adult calorie intake independently and additively affect mitogenic signaling and increase mammary tumor susceptibility. Carcinogenesis 2010;31:1873–81. http://dx.doi.org/10.1093/carcin/bgq095.

[40] Cui Y, Vogt S, Olson N, Glass AG, Rohan TE. Levels of zinc, selenium, calcium, and iron in benign breast tissue and risk of subsequent breast cancer. Cancer Epidemiol Biomarkers Prev 2007;16:1682–5. http://dx.doi.org/10.1158/1055-9965.EPI-07-0187.

[41] Prasad AS, Beck FW, Endre L, Handschu W, Kukuruga M, Kumar G. Zinc deficiency affects cell cycle and deoxythymidine kinase gene expression in HUT-78 cells. J Lab Clin Med 1996;128:51–60.

[42] Paski SC, Xu Z. Growth factor stimulated cell proliferation is accompanied by an elevated labile intracellular pool of zinc in 3T3 cells. Can J Physiol Pharmacol 2002; 80:790–5.
[43] Kloubert V, Rink L. Zinc as a micronutrient and its preventive role of oxidative damage in cells. Food Funct 2015;6:3195–204. http://dx.doi.org/10.1039/c5fo00630a.
[44] Maret W, Sandstead HH. Zinc requirements and the risks and benefits of zinc supplementation. J Trace Elem Med Biol 2006;20(1):3–18. http://dx.doi.org/10.1016/j.jtemb.2006.01.006.
[45] Fosmire GJ. Zinc toxicity. Am J Clin Nutr 1990;51:225–7.
[46] Rudolf E, Cervinka M. Depletion of endogenous zinc stores induces oxidative stress and cell death in human melanoma cells. Acta Medica (Hradec Králové) 2004;47:91–6.
[47] Jayaraman AK, Jayaraman S. Increased level of exogenous zinc induces cytotoxicity and up-regulates the expression of the ZnT-1 zinc transporter gene in pancreatic cancer cells. J Nutr Biochem 2011;22:79–88. http://dx.doi.org/10.1016/j.jnutbio.2009.12.001.
[48] Gumulec J, Masarik M, Krizkova S, Adam V, Hubalek J, Hrabeta J, et al. Insight to physiology and pathology of zinc(II) ions and their actions in breast and prostate carcinoma. Curr Med Chem 2011;18:5041–51.
[49] Cai Q, Shu X-O, Wen W, Cheng J-R, Dai Q, Gao Y-T, et al. Genetic polymorphism in the manganese superoxide dismutase gene, antioxidant intake, and breast cancer risk: results from the shanghai breast cancer study. Breast Cancer Res 2004;6: R647–55. http://dx.doi.org/10.1186/bcr929.
[50] Akagi T, Motegi M, Tamura A, Suzuki R, Hosokawa Y, Suzuki H, et al. A novel gene, MALT1 at 18q21, is involved in t(11;18) (q21;q21) found in low-grade B-cell lymphoma of mucosa-associated lymphoid tissue. Oncogene 1999;18:5785–94.
[51] Ruland J, Duncan GS, Elia A, del Barco Barrantes I, Nguyen L, Plyte S, et al. Bcl10 is a positive regulator of antigen receptor-induced activation of NF-kappaB and neural tube closure. Cell 2001;104:33–42.
[52] Choi HJ, Lee JM, Kim H, Nam HJ, Shin H-JR, Kim D, et al. Bcl3-dependent stabilization of CtBP1 is crucial for the inhibition of apoptosis and tumor progression in breast cancer. Biochem Biophys Res Commun 2010;400:396–402. http://dx.doi.org/10.1016/j.bbrc.2010.08.084.
[53] Massoumi R, Chmielarska K, Hennecke K, Pfeifer A, Fässler R. Cyld inhibits tumor cell proliferation by blocking Bcl-3-dependent NF-kappaB signaling. Cell 2006; 125:665–77. http://dx.doi.org/10.1016/j.cell.2006.03.041.
[54] Yeh PY, Kuo S-H, Yeh K-H, Chuang S-E, Hsu C-H, Chang WC, et al. A pathway for tumor necrosis factor-alpha-induced Bcl10 nuclear translocation. Bcl10 is up-regulated by NF-kappaB and phosphorylated by Akt1 and then complexes with Bcl3 to enter the nucleus. J Biol Chem 2006;281:167–75. http://dx.doi.org/10.1074/jbc.M511014200.
[55] Maldonado V, Melendez-Zajgla J. Role of Bcl-3 in solid tumors. Mol Cancer 2011; 10:152. http://dx.doi.org/10.1186/1476-4598-10-152.
[56] Kashatus D, Cogswell P, Baldwin AS. Expression of the Bcl-3 proto-oncogene suppresses p53 activation. Genes Dev 2006;20:225–35. http://dx.doi.org/10.1101/gad.1352206.
[57] Wakefield A, Soukupova J, Montagne A, Ranger J, French R, Muller WJ, et al. Bcl3 selectively promotes metastasis of ERBB2-driven mammary tumors. Cancer Res 2013;73:745–55. http://dx.doi.org/10.1158/0008-5472.CAN-12-1321.
[58] Westerheide SD, Mayo MW, Anest V, Hanson JL, Baldwin AS. The putative oncoprotein Bcl-3 induces cyclin D1 to stimulate G(1) transition. Mol Cell Biol 2001;21:8428–36. http://dx.doi.org/10.1128/MCB.21.24.8428-8436.2001.
[59] Heissmeyer V, Krappmann D, Wulczyn FG, Scheidereit C. NF-kappaB p105 is a target of IkappaB kinases and controls signal induction of Bcl-3-p50 complexes. EMBO J 1999;18:4766–78. http://dx.doi.org/10.1093/emboj/18.17.4766.
[60] Brenne A-T, Fagerli U-M, Shaughnessy JD, Våtsveen TK, Rø TB, Hella H, et al. High expression of BCL3 in human myeloma cells is associated with increased proliferation and inferior prognosis. Eur J Haematol 2009;82:354–63. http://dx.doi.org/10.1111/j.1600-0609.2009.01225.x.
[61] Rebollo A, Dumoutier L, Renauld JC, Zaballos A, Ayllón V, Martínez-A C. Bcl-3 expression promotes cell survival following interleukin-4 deprivation and is controlled by AP1 and AP1-like transcription factors. Mol Cell Biol 2000;20: 3407–16.
[62] Elliott SF, Coon CI, Hays E, Stadheim TA, Vincenti MP. Bcl-3 is an interleukin-1-responsive gene in chondrocytes and synovial fibroblasts that activates transcription of the matrix metalloproteinase 1 gene. Arthritis Rheum 2002;46: 3230–9. http://dx.doi.org/10.1002/art.10675.
[63] Hu X, Nesic-Taylor O, Qiu J, Rea HC, Fabian R, Rassin DK, et al. Activation of nuclear factor-kappaB signaling pathway by interleukin-1 after hypoxia/ischemia in neonatal rat hippocampus and cortex. J Neurochem 2005;93:26–37. http://dx.doi.org/10.1111/j.1471-4159.2004.02968.x.
[64] Brocke-Heidrich K, Ge B, Cvijic H, Pfeifer G, Löffler D, Henze C, et al. BCL3 is induced by IL-6 via Stat3 binding to intronic enhancer HS4 and represses its own transcription. Oncogene 2006;25:7297–304. http://dx.doi.org/10.1038/sj.onc.1209711.
[65] Kuwata H, Watanabe Y, Miyoshi H, Yamamoto M, Kaisho T, Takeda K, et al. IL-10-inducible Bcl-3 negatively regulates LPS-induced TNF-alpha production in macrophages. Blood 2003;102:4123–9. http://dx.doi.org/10.1182/blood-2003-04-1228.
[66] Valenzuela JO, Hammerbeck CD, Mescher MF. Cutting edge: Bcl-3 up-regulation by signal 3 cytokine (IL-12) prolongs survival of antigen-activated CD8 T cells. J Immunol 2005;174:600–4.
[67] Clarkson RW, Heeley JL, Chapman R, Aillet F, Hay RT, Wyllie A, et al. NF-kappaB inhibits apoptosis in murine mammary epithelia. J Biol Chem 2000;275: 12737–42.
[68] Acharyya S, Oskarsson T, Vanharanta S, Malladi S, Kim J, Morris PG, et al. A CXCL1 paracrine network links cancer chemoresistance and metastasis. Cell 2012;150: 165–78. http://dx.doi.org/10.1016/j.cell.2012.04.042.
[69] Pecot CV, Rupaimoole R, Yang D, Akbani R, Ivan C, Lu C, et al. Tumour angiogenesis regulation by the miR-200 family. Nat Commun 2013;4:2427. http://dx.doi.org/10.1038/ncomms3427.
[70] Zou A, Lambert D, Yeh H, Yasukawa K, Behbod F, Fan F, et al. Elevated CXCL1 expression in breast cancer stroma predicts poor prognosis and is inversely associated with expression of TGF-β signaling proteins. BMC Cancer 2014;14:781. http://dx.doi.org/10.1186/1471-2407-14-781.
[71] Youngs SJ, Ali SA, Taub DD, Rees RC. Chemokines induce migrational responses in human breast carcinoma cell lines. Int J Cancer 1997;71:257–66.
[72] Loukinova E, Dong G, Enamorado-Ayalya I, Thomas GR, Chen Z, Schreiber H, et al. Growth regulated oncogene-alpha expression by murine squamous cell carcinoma promotes tumor growth, metastasis, leukocyte infiltration and angiogenesis by a host CXC receptor-2 dependent mechanism. Oncogene 2000;19:3477–86. http://dx.doi.org/10.1038/sj.onc.1203687.
[73] Wang X, Zhou B. Dietary zinc absorption: a play of zips and ZnTs in the gut. IUBMB Life 2010;62(3):176–82. http://dx.doi.org/10.1002/iub.291.
[74] Liuzzi JP, Blanchard RK, Cousins RJ. Differential regulation of zinc transporter 1, 2, and 4 mRNA expression by dietary zinc in rats. J Nutr 2001;131(1):46–52.
[75] Chung CS, Stookey J, Dare D, Welch R, Nguyen TQ, Roehl R, et al. Current dietary zinc intake has a greater effect on fractional zinc absorption than does longer term zinc consumption in healthy adult men. Am J Clin Nutr 2008;87(5): 1224–9.