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

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ABSTRACT

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.

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 hematoxilin 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