Reproductive Toxicology 54 (2015) 84–92

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Paradoxical zinc toxicity and oxidative stress in the mammary gland during marginal dietary zinc deficiency

Zeynep Bostanci[a,d], Ronald P. Mack Jr[a,b], Sooyeon Lee[a,c], David I. Soybel[a,d,e], Shannon L. Kelleher[a,c,d,e,*]

[a] Department of Nutritional Sciences, The Pennsylvania State University, United States
[b] Department of Kinesiology, The Pennsylvania State University, United States
[c] Interdisciplinary Graduate Program in Physiology, The Pennsylvania State University, United States
[d] Department of Surgery, Penn State Hershey College of Medicine, United States
[e] Department of Cell and Molecular Physiology, Penn State Hershey College of Medicine, United States

ARTICLE INFO

Article history:
Received 11 February 2014
Received in revised form 7 July 2014
Accepted 22 July 2014
Available online 1 August 2014

Keywords:
Mammary gland
Zinc transporter
Oxidative stress
Collagen
Estrogen receptor
Breast cancer
Ductal hyperplasia

ABSTRACT

Zinc (Zn) regulates numerous cellular functions. Zn deficiency is common in females; ~80% of women and 40% of adolescent girls consume inadequate Zn. Zn deficiency enhances oxidative stress, inflammation and DNA damage. Oxidative stress and inflammation is associated with breast disease. We hypothesized that Zn deficiency increases oxidative stress in the mammary gland, altering the microenvironment and architecture. Zn accumulated in the mammary glands of Zn deficient mice and this was associated with macrophage infiltration, enhanced oxidative stress and over-expression of estrogen receptor α. Ductal and stromal hypercellularity was associated with aberrant collagen deposition and disorganized e-cadherin. Importantly, these microenvironmental alterations were associated with substantial impairments in ductal expansion and mammary gland development. This is the first study to show that marginal Zn deficiency creates a toxic microenvironment in the mammary gland impairing breast development. These changes are consistent with hallmarks of potential increased risk for breast disease and cancer.

1. Introduction

Zinc (Zn) is a critical catalytic, structural and regulatory cofactor required by over 10% of the eukaryotic proteome and plays a vital role in >300 cellular functions including apoptosis, cell signaling, proliferation, differentiation, motility and antioxidant defense (reviewed in [1]). Cellular Zn metabolism is regulated by two families of Zn transporters that either import (ZIP proteins) or export (ZnT proteins) Zn from the cytoplasm (reviewed in [2,3]). Inability to tightly control Zn accumulation or depletion is cytotoxic, resulting in mitochondrial dysfunction, elevated production of reactive oxygen species (ROS), inflammation and apoptosis [4,5] *in vitro*. Paradoxically, Zn deficiency *in vivo* is cytotoxic, increasing oxidative stress in liver [6,7] and kidney [8]. Zn deficiency in male rats increases oxidative stress and DNA damage in testes [9,10] and prostate [11]. These considerations suggest that Zn deficiency causes organ dysfunction by increasing oxidative stress in tissues, especially in metabolically active organs such as liver or kidney.

The consequences of Zn deficiency are of global concern, particularly in women of child-bearing years. Recently reports indicate that, even in developed countries including the US and Canada, adolescent girls are at risk for Zn deficiency, with ~40% of young girls consuming diets containing inadequate Zn [12,13]. The effects of Zn deficiency on mammary gland development and function are not known. In addition, it is estimated that ~80% of women of reproductive age are at risk for Zn deficiency due to low intake of bioavailable Zn and increased demand for Zn during both pregnancy and lactation [12,14,15].

Mammary gland function is driven by numerous hormones, growth factors and cytokines, including estrogen, leptin and prolactin (reviewed in [16]) and has numerous specialized requirements for Zn during pubertal and reproductive development (reviewed in [1]). Several lines of evidence indicate that the inability to appropriately manage Zn in the mammary gland compromises breast function [17–22]. Our previous studies found that even a marginal Zn deficiency during the peri-natal period in rodents substantially impairs mammary gland function, compromises alveolar

* Corresponding author at: Departments of Nutritional Sciences, Surgery and Cell and Molecular Physiology, Penn State Hershey College of Medicine, 500 University Ave, Hershey, PA 17033, United States. Tel.: +1 717 531 1778; fax: +1 717 531 0884.
E-mail address: slk39@psu.edu (S.L. Kelleher).

http://dx.doi.org/10.1016/j.reprotox.2014.07.076
0890-6238/

development and secretory capacity and alters milk composition during lactation [23,24]. Moreover, Adzersen et al. [25] found a significant inverse association between dietary Zn and breast cancer risk and low red blood cell Zn levels were observed in women with breast cancer [26]. We and others have shown that Zn hyperaccumulates in breast tumors [27–29], and breast tumor cell lines [30]; which has been associated with increased expression of Zn transporters such as ZnT2 [30], ZIP6 [31] and ZIP10 [32]. These considerations suggest that Zn deficiency and ensuing alterations in cellular Zn management may lead to the inability of the breast to expand during pregnancy, function properly during lactation, and conform to the normal schedule of involution.

Herein, we explore the hypothesis that marginal Zn deficiency increases oxidative stress and alters tissue architecture in the developing mammary gland. We report that diet-induced, marginal Zn deficiency results in oxidative stress and macrophage infiltration in the mammary gland. This creates a toxic microenvironment driven by increased expression of key Zn transporters resulting in Zn hyperaccumulation and is associated with increased estrogen receptor α (ERα) expression, alterations in ductal organization, impaired microarchitecture and enhanced mammary gland fibrosis, all of which are consistent with hallmarks of potential increased risk of breast disease and cancer. Collectively, our work suggests that inadequate Zn intake leads, paradoxically, to Zn toxicity in the mammary gland, thereby impairing lactation and increasing susceptibility to breast diseases characterized by inflammation. Our findings offer the possibility that such mild systemic deficiencies in Zn pose a more general threat to reproductive health in women.

2. Materials and methods

2.1. Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University, which is accredited by the American Association for the Accreditation of Laboratory Animal Care. Four-week old, nulliparous female C57/Bl6 mice were obtained commercially (Harlan Sprague Dawley, Indianapolis, IN). Mice were housed in polycarbonate cages and after a 7 d acclimation period, were fed a purified diet (MP Biomedical, Santa Ana, CA) containing adequate (30 mg Zn/kg diet; ZA) or marginally deficient (15 mg Zn/kg diet; ZD) Zn levels *ad libitum* for ∼20 weeks ($n = 10$ mice/diet). A long-term model of marginal Zn deficiency was chosen to mirror the consequences of chronic low Zn intake in humans. Zn content of the diets was confirmed by atomic absorbance spectroscopy. Mice were maintained on a 12 h light/dark cycle under controlled temperature and humidity. The mice were euthanized by CO_2 asphyxiation. Blood was drawn by cardiac puncture and collected into heparinized tubes and plasma was separated by centrifugation at 1000 × *g* for 15 min at 4 °C. Mammary glands were excised and mounted on glass slides for morphological analysis, fixed in 4% phosphate-buffered paraformaldehyde overnight, or stored at −80 °C until analysis.

2.2. Whole mount imaging

Excised axillary mammary glands from four mice/group were mounted on glass slides and fixed with Carnoy's fixative (60% ethanol, 30% chloroform and 10% glacial acetic acid) overnight. The tissues were rehydrated in decreasing concentrations of ethanol, rinsed in water for 5 min and then stained overnight with 0.2% carmine powder (Sigma–Aldrich, St. Louis, MO) and 0.5% aluminum potassium sulfate (Sigma–Aldrich). The mammary glands were dehydrated using a graded ethanol series and the fat pads were cleared in xylenes for 60 min [33]. Whole mounts were viewed at 0.5–40× magnification using Leica DM IL LED microscope attached with a Leica DFC425 digital camera and images were collected using Leica Application Suite (V3.6). To measure the size of terminal ductal structures, images of whole mount mammary glands at 40× magnification were analyzed using Adobe Photoshop CS3 (Extended V10.0). Distinct terminal ductal structures were selected using the Magic Wand Tool and the area (μm^2) was recorded after setting the measurement scale to 200 pixels as 200 μm. Three different images/mammary gland were captured from four different mice and the size of the terminal ductal structures was averaged for statistical analysis. To measure the number of terminal ductal structures, images of whole mount mammary glands at 4× magnification were analyzed and distinct terminal ductal structures were selected using the Count Tool. Two different images/mammary glands were captured from four different mice and the number of terminal ductal structures was averaged for statistical analysis. To determine the degree of mammary gland expansion, images of whole mount inguinal mammary glands were collected at 0.5× magnification and analyzed using Adobe Photoshop C3S (Extended V10.0). The Lasso Tool was utilized to trace the perimeter of the mammary fat pad and the most distal-reaching ductal structures along the perimeter of the ductal network. Once the perimeter was traced, total pixel area was calculated. Total ductal infiltration was then calculated as a ratio of ductal tree pixel area relative to total mammary gland fat pad pixel area. To determine the number of secondary and tertiary branches in the mammary glands, images of whole mount axillary mammary glands were collected at 4× magnification and analyzed using Adobe Photoshop C3S (Extended V10.0). Distinct branching points were selected using the Count Tool from two different areas per mammary gland from four different mice and the number of distinct branch points was averaged for statistical analysis.

2.3. Histology

Excised inguinal mammary glands were fixed in 4% phosphate-buffered paraformaldehyde overnight at 4 °C. Fixed glands were washed three times for 30 min in phosphate-buffered saline (PBS) and three times for 30 min in 70% ethanol at 4 °C. The glands were embedded in paraffin and 5 μm sections were adhered to positively-charged glass slides. *Hematoxylin and eosin (H&E) staining* ($n = 10$/group) – sections were stained with H&E as previously described [24]. *Trichrome staining* ($n = 4$/group) – sections were deparaffinized in xylenes and rehydrated through 100%, 70%, and 50% ethyl alcohol. Sections were incubated in Bouin's solution for 1 h at 56 °C, stained in Weigert's iron hematoxylin, Biebrich scarlet-acid fuchsin, and then rinsed in phosphomolybdic-phosphotungstic acid (1%). Slides were then stained in aniline blue and returned to phosphomolybdic-phosphotungstic acid (1%). Sections were washed in acetic acid (1%) and serially dehydrated through 100%, 95% ethyl alcohol and xylenes. *Immunohistochemistry* – antibodies used for immunostaining were as follows: anti-mouse F4/80 (macrophage marker, 1:1000; AbD Serotech, Kidlington, UK), anti-mouse e-cadherin (1:1000; Abcam, Cambridge, MA), anti-4-hydroxy-2-nonenal (4HNE, 1:100; Abcam) and anti-8-hydroxydeoxyguanosine (8-OHDG) (1:50; Abcam). F4/80, e-cadherin and 4HNE was detected using the Vectastain ABC Kit as described previously and counterstained with toluidine blue [34]. 8-OHDG was detected by immunofluorescence using anti-mouse IgG Alexa Fluor 488. Sections were incubated with rabbit IgG (1:100) instead of anti-4HNE or anti-8-OHDG as negative controls. Sections were imaged using a Leica DM IL LED microscope and LAS V3.6 software. Sections were viewed at 4×, 10× or 40× magnification using Leica DM IL LED microscope attached with a Leica DFC425 digital camera and images were collected using Leica Application Suite (V3.6). Three different images/mammary glands were