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

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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.

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Disclosures

The authors have nothing to disclose.

Author contributions

ZB and SLK conception and research design; ZB, RM and SL performed experiments and analyzed data; ZB, DIS and SLK interpreted the results; ZB and SLK wrote manuscript; ZB, DIS and SLK edited and revised manuscript; ZB, RM, SL, DIS and SLK approved final version of manuscript.

Keywords

mammary gland; zinc transporter; oxidative stress; collagen; estrogen receptor; breast cancer; ductal hyperplasia

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) 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 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 (ER α) 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 wks (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 CO₂ asphyxiation. Blood was drawn by cardiac puncture and collected into heparinized tubes and plasma was separated by centrifugation at $1,000 \times g$ 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 4 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 0.5-40X 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 40X 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 4 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 4X magnification were analyzed and distinct terminal ductal structures were selected using the Count Tool. Two different images/mammary gland were captured from 4 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 0.5X 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 4X magnification and analyzed using Adobe Photoshop C3S (Extended V10.0). Distinct branching points were selected using the Count Tool from 2 different areas per mammary gland from 4 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 4X, 10X or 40X 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 gland were captured from 3 independent mice and the number of cells stained positive for F4/80 or 8-OHDG were counted and expressed as mean number of cells $\pm$ SD.

2.4 Matrix metalloproteinase (MMP) activity

Gel zymography was used to assess MMP activity. This method was adapted from [35]. Protein lysates (100 µg) were diluted in non-reducing SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 4% SDS, and 0.001% bromophenol blue) and separated on 10% SDS-PAGE gels containing 1 mg/mL gelatin (Bio-Rad, Hercules, CA). After electrophoresis, the gels were washed for 1 h at room temperature in renaturing buffer (10 mM Tris-HCl, pH 7.5, 2.5% Triton X-100), incubated for 15 h at 37 °C in enzyme buffer (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 5 mM CaCl₂, 0.02% Brij-35), and stained with Coomassie Brilliant Blue R-250. MMP activity was detected after destaining by identification of clear bands over blue background. Gels were imaged with Odyssey® CLX imaging system (LI-COR Biosciences, Lincoln, NE) and quantification was performed on Odyssey Image Studio (Ver 2.0). Data represent mean area of cleared bands ± SD.

2.5 Zn Analysis

Zn concentrations of diet, plasma, liver and mammary gland (n=10/group) were determined by atomic absorption spectrophotometry (AAnalyst 400, Perkin Elmer; Waltham, MA) as previously described [15].

2.6 Plasma leptin

Plasma leptin concentration was measured using a commercially available kit (SIGMA-Aldrich) per manufacturer's instructions. Data represents mean plasma leptin (nmol/L) ± SD.

2.7 Immunoblotting

Tissue homogenates were generated by homogenizing mammary gland (~100 mg) in homogenization buffer (HEPES (10 mmol/L, pH 7.5), mannitol (210 mmol/L), sucrose (70 mmol/L), and EDTA (1 mmol/L)) and centrifuged for 5 min at 500 × g. Crude membrane fractions were generated as previously described [24]. Equal amounts (20-60 µg) of tissue homogenate (e-cadherin, smooth muscle actin [αSMA], MMP2 and ERα) or membrane proteins (ZIP6, ZIP10 and ZnT2) were diluted 1:1 in Laemmli sample buffer with DTT and resolved by SDS-PAGE (10%) at 200 V for 1 hr. Proteins were transferred to nitrocellulose membrane at 100V for 1 hr. The membrane was blocked in 1% bovine serum albumin for 1 h at room temperature and then incubated simultaneously with primary antibody (e-cadherin and αSMA: 1:1000, Abcam, Cambridge, MA; MMP2 1:2000, Abcam; ERα: 1:1000, Abcam; ZIP6/LIV- 1:1:1000, Santa Cruz Biotechnology; ZIP10: 1:1000, ProSci, Poway, CA; ZnT2: 1:1000, [36]; and β-actin antibody (1:5000; Sigma-Aldrich) as loading control overnight at 4 °C. Membranes were washed three times for 5 min in PBS with 0.1% Tween (PBS-T) and then incubated for 1 h at room temperature with infrared secondary antibody: IRDye® 800CW goat anti-rabbit IgG and/or IRDye® 680RD goat anti-mouse IgG (LICOR Biosciences) diluted 1:20,000 in LI-COR blocking buffer. The membranes were washed three times for 5 min in PBS-T and rinsed in PBS before scanning on the Odyssey® CLX imaging system (LI-COR Biosciences). Band density was measured on Odyssey Image Studio (Ver 2.0). Data represent mean ratio of band density to β-actin, normalized to background fluorescence ± SD.

2.8 Statistical analysis

Results are presented as mean $\pm$ SD. Statistical comparisons were performed using student's *t*-test (Prism GraphPad, Berkeley, CA) and a significant difference was demonstrated at $p < 0.05$.

3. Results

3.1 Marginal Zn intake is unrelated to plasma Zn levels or weight gain

The majority of animal studies exploring effects of Zn intake utilize a short-term (<30 d) and severe model of Zn restriction (<1 mg Zn/kg diet). However, because severe Zn restriction is not generally relevant to deficiency states in humans, we utilized a chronic model (~ 20 wk) of marginal Zn restriction (15 mg Zn/kg). Importantly, Zn restriction began at 5 wk of age, around the onset of puberty which begins ~ 5 wk in C57Bl/6 mice [37]. Our restriction paradigm did not significantly affect weight gain (ZA, $8.7 \text{ g} \pm 1.7$; ZD, $7.4 \text{ g} \pm 1.3$). Consistent with our previous studies using a similar marginal Zn restriction paradigm [23,24,38], marginal Zn restriction in this study did not significantly lower plasma Zn concentration (ZA, $10.2 \text{ } \mu\text{M} \pm 1.1$; ZD, $9.4 \text{ } \mu\text{M} \pm 1.0$). These observations confirm that our dietary paradigm did not result in a clinically severe Zn deficiency. Recently, leptin has been shown to be a critical regulator of mammary gland development [16]. While limited amounts of tissue precluded the measurement of leptin in the mammary gland, we found that plasma leptin concentration was significantly lower in mice fed ZD ($32 \text{ nM} \pm 21$) compared with mice fed ZA ($175 \text{ nM} \pm 150$, $p < 0.05$).

3.2 Marginal Zn intake is associated with oxidative stress, macrophage infiltration and collagen deposition in the mammary gland

To determine if marginal Zn intake was associated with increased oxidative stress in the mammary gland, we used 4HNE [39] and 8-OHDG [40] as markers of oxidative damage. We noted that 4HNE staining was more pronounced in mice fed a ZD diet compared with mice fed a ZA diet (Figure 1). As a second marker of oxidative stress, we found that mice fed ZD had significantly more 8-OHDG positive nuclei ($36 \text{ nuclei/field} \pm 16$) compared with mice fed ZA ($12 \text{ nuclei/field} \pm 4$, $p < 0.05$). We noted that increased oxidative stress was associated with accumulation of acellular fibrotic tissue that we identified as collagen, deposited throughout the stroma (Figure 1) in the mammary glands from ZD mice. Finally, we used immunohistochemistry to quantify the number of macrophages using F4/80 as a marker. We detected greater macrophage infiltration in mice fed a ZD diet ($22.63 \text{ macrophages/area} \pm 6.5$) compared with mice fed ZA ($10.4 \text{ macrophages/area} \pm 3.5$, $p < 0.05$).

3.3 Oxidative stress is associated with Zn accumulation in the mammary gland

Previous studies observed that oxidative stress is associated with Zn accumulation in liver [41] and adipose tissue [42]. Similarly, in the current set of studies, we found that oxidative stress was associated with greater Zn accumulation in the mammary gland of mice fed ZD ($6.4 \text{ } \mu\text{g Zn/g} \pm 2.3$) compared with mice fed a ZA diet ($3.7 \text{ } \mu\text{g Zn/g} \pm 2.0$, $p < 0.05$). Hyperaccumulation of Zn was not specific to the mammary gland as similar observations

were made in the liver (ZD: $26.6 \mu\text{g Zn/g} \pm 1.3$; ZA: $24.3 \mu\text{g Zn/g} \pm 0.6$, $p < 0.001$). Consistent with aberrant Zn accumulation in the mammary gland, we found that the abundance of the Zn import proteins ZIP6 and ZIP10 were both significantly higher in mice fed a ZD diet compared with mice fed a ZA diet (Figure 2A-B). Expression of ZnT2 is critical for vesicular Zn sequestration and protection from Zn toxicity [30]. Consistent with Zn accumulation in the mammary gland, we found that ZnT2 expression was significantly higher in mammary glands from mice fed a ZD diet (Figure 2C). This suggests that Zn hyperaccumulation in the mammary gland is at least partially driven by increased Zn uptake through ZIP6 and ZIP10 and that Zn is sequestered into vesicles through a ZnT2-mediated mechanism.

3.4 The toxic microenvironment in the mammary gland is associated with substantially impaired architecture

Previously we found that marginal Zn deficiency reduces secretory capacity by 80% during lactation [24]. One hypothesis is that marginal Zn deficiency results in insufficient mammary gland expansion prior to lactation. To test this hypothesis, we first analyzed mammary gland whole mounts and visually noted that the development of secondary and tertiary branches was sparser in mice fed ZD compared with mice fed ZA (Figure 3). The ratio of ductal area/fat pad area in mice fed ZD (0.74 ± 0.06) was significantly less than in mice fed ZA (0.87 ± 0.05 , $p < 0.05$). In addition, the mean number of terminal ductal structures in mice fed ZD ($28.6 \text{ terminal ductal structures}/5 \text{ mm}^2 \text{ area} \pm 19.4$) tended to be lower than in mice fed ZA ($59.4 \text{ terminal ductal structures}/5 \text{ mm}^2 \text{ area} \pm 22.9$, $p = 0.08$) and the terminal ductal structure area in mice fed ZD ($2067 \mu\text{m}^2 \pm 427$) was significantly smaller than in mice fed ZA ($3011 \mu\text{m}^2 \pm 235$, $p < 0.01$). We recently showed that matrix metalloproteinase 2 (MMP2) activity is reduced by over-expressing ZnT2 [35]. Because ZnT2 was over-expressed in the mammary glands of ZD mice and MMP2 is a Zn-dependent protease that is critical for mammary gland expansion and development [43], we assessed mammary gland MMP2 activity. Consistent with our previous study and defects observed in duct elongation and supernumerary branch development in the current study, mice fed a ZD diet had ~30% lower MMP2 activity (Figure 4) while no effect on MMP2 abundance was detected (data not shown). Mammary gland sections stained with H&E revealed several key defects in morphology (Figure 5). We noted the presence of acellular tissue accumulation which was previously determined to be collagen (Section 3.1) and epithelial hypercellularity in the mammary glands from mice fed a ZD diet which was not seen in the mammary glands from mice fed ZA (Figure 5A-B). While the ductal epithelium of mice fed a ZA diet was well organized and the duct lumens were patent (Figure 5C), we noted that the ductal epithelium of mice fed a ZD diet was disorganized and the duct lumens were collapsed (Figure 5D).

Oxidative stress alters expression of vital structural proteins such as αSMA [44] and e-cadherin [45]. Thus, we measured the abundance of e-cadherin, a cell adhesion protein found on the basal membrane of differentiated luminal epithelial cells and αSMA , a marker of differentiated myoepithelial cells. There was no significant effect on total e-cadherin abundance (data not shown); however, consistent with effects of oxidative stress-derived hyperplasia [45], e-cadherin staining (Figure 5E, F) further confirmed epithelial hyperplasia

and the disorganized ductal structure from mice fed a ZD diet (Figure 5F) compared with mice fed a ZA diet (Figure 5E). Total α SMA abundance was modestly lower in mice fed a ZD diet compared with mice fed a ZA diet (Figure 6A).

Moreover, oxidative stress results in hyperplasia in the mammary gland with premalignant features [45]. In addition, the over-expression of ER α in transgenic mice results in ductal hyperplasia [46]. Therefore we assessed ER α expression in tissue homogenates. We found that mice fed a ZD diet had ~2-fold greater ER α expression compared with mice fed a ZA diet, $p < 0.05$ (Figure 6B).

4. Discussion

In the present study we show that marginal Zn deficiency paradoxically drives tissue hyper-accumulation of Zn and creates a toxic microenvironment in the mammary glands of female mice. The downstream effects include oxidative stress, increased macrophages, disturbances in markers of orderly gland architecture and expansion, and increased expression of estrogen receptors that have the potential to drive the appearance of a pre-malignant state. Importantly this results in numerous developmental defects that could impair lactation and ultimately affect breast health. Herein, we utilized a marginal Zn restriction paradigm which more closely represents chronic, suboptimal Zn intake in adolescent girls and reproductive age women, as opposed to a severe Zn restriction model that is usually employed. Utilizing a pre-clinical model, our studies provide a rationale for studies of the clinical effects of sub-optimal Zn intake on breast development or health in women. Our results suggest that marginal Zn deficiency may have important implications on female reproductive health.

Consistent with reports associating severe Zn deficiency with oxidative stress and inflammation in other tissues [6-9,11,47], we found that marginal Zn deficiency was also associated with oxidative stress and inflammation in both the ductal epithelium and adipocyte-rich stroma in the mammary gland. An important component of the stroma is the protein collagen. Excess collagen directly inhibits mammary gland expansion [48] and has major implications for breast disease risk [49]. Consistent with reports that Zn deficiency increases inflammation and fibrosis in lung [47], we observed enhanced collagen accumulation, primarily in the peri-ductal region, associated with Zn hyperaccumulation and oxidative stress in Zn deficient mice. This likely created a physical barrier that impeded ductal elongation in addition to altering the microenvironment of the mammary gland. Collagen deposition in response to oxidative stress may be potentiated through the activation of cell signaling cascades such as Src [50] and has been linked to fibrosis in liver [51]. This is particularly relevant as breast fibrosis, a component of fibrocystic breast disease which is a painful condition associated with breast inflammation, afflicts about 50% of all women and is most common during the reproductive years. In addition, fibrotic (or dense) breast tissue increases a woman's risk for breast cancer [49]. Further studies are required to understand effects of Zn deficiency on regulatory mechanisms through which the stroma is remodeled during breast development and is dysregulated in breast disease.

Although oxidative stress increases Zn levels in liver [41] and adipose [42], to our knowledge our study is the first to report marginal Zn intake as an underlying driver of

oxidative stress concomitant with Zn hyperaccumulation. The combination is particularly relevant in the mammary gland because both oxidative stress [52] and Zn hyperaccumulation [26] in breast tissue are associated with dysfunction and disease in women. To identify mechanisms that may be responsible for Zn hyperaccumulation in response to Zn deficiency, we specifically chose to delineate effects on ZIP6, ZIP10 and ZnT2 as they are overexpressed in breast cancer [30-32]. While numerous Zn transporters may have been affected [34], we found that Zn hyperaccumulation was indeed associated with increased expression of ZIP6 and ZIP10, suggesting they play a role in Zn uptake from systemic circulation. ZIP6 expression may have been driven by augmented expression of ER α as ZIP6 is predicted to be an estrogen-regulated gene [53]. However, both glucose and calcium signaling also increase ZIP6 expression [54], and thus other regulatory factors cannot be ruled out. Recent work by Hogstrand et al [55] found that ZIP6 over-expression drives cell motility and promotes epithelial-to-mesenchymal transition in MCF7 breast cancer cells. In contrast, ZIP6 over-expression is associated with better prognosis in ER⁺ breast disease [31], and our previous work found that repressing ZIP6 expression in ER⁺ tumorigenic T47D breast cancer cells promotes epithelial-to-mesenchymal transition [56]. Therefore the biological implications of ZIP6 overexpression are not currently understood. In addition to ZIP6, we noted that Zn hyperaccumulation was associated with ZIP10 overexpression. ZIP10 expression is associated with cell migration in ER⁻/PR⁻/HER2⁻ invasive MDA-MB-231 breast cancer cells [32]. ZIP10 over-expression in mammary glands enriched in Zn was an intriguing finding as ZIP10 is normally repressed by excess Zn through a metal transcription factor-1 (MTF-1) mediated response [57]. It is possible that ZIP10 expression and Zn accumulation occur in different cell-types or that Zn accumulation is not accessible to ZIP10 regulatory mechanisms. Alternatively, factors other than Zn such as oxidative stress [58,59] may be responsible for driving ZIP10 expression and dysregulating normal Zn homeostatic mechanisms. Consistent with a role for ZnT2 protecting cells from Zn toxicity by sequestering Zn into vesicles [30], Zn hyperaccumulation in the mammary glands from Zn deficient mice was associated with increased expression of ZnT2. This seems reasonable given the role of Zn in positively regulating ZnT2 expression due to the presence of metal responsive elements in the promoter of the gene that encodes ZnT2 (*SLC30A2*) [60]. Alternatively, ZnT2 expression is increased by nitric oxide [61], suggesting that enhanced oxidative stress may have contributed to increased ZnT2 expression. Whether oxidative stress drives Zn accumulation or if Zn accumulation enhances oxidative stress is currently not understood, but clearly the combination perpetuates a toxic microenvironment in the mammary gland.

Zn hyperaccumulation, oxidative stress and inflammation were associated with impaired mammary gland development. Such defects are associated with reduced α SMA which may reflect reduced number of epithelial cells or compromised balance between luminal and myoepithelial cells. Moreover, such defects may underlie compromised mammary gland function that we previously observed during lactation [24] and may also suggest increased susceptibility to mitogenic and mutagenic effects of oxidative stress. Our observation that activity of the Zn-dependent enzyme MMP2 was lower in the mammary glands of Zn deficient mice sheds some mechanistic light on our observations as MMP2 is a Zn-dependent protein and is critical for ductal elongation and infiltration into the mammary fat

pad [43]. Thus, ZnT2-mediated Zn sequestration into vesicles may have reduced the metallation and activation of MMP2 as previously described [35]. However, we cannot rule out effects on decreased activity of other MMP family members (currently there are 24) or increased activity of tissue inhibitors of metalloproteinases (TIMPs) as has been reported in Caco-2 cells exposed to reduced bioavailable Zn [62]. Alternatively, Zn deficiency may affect key hormones important for mammary gland development. Kwun et al [63] found that marginal Zn deficiency in rats decreased leptin expression in white adipose. Although, we were unable to quantify leptin levels in the mammary gland due to the limited amount of tissue, we did find that marginal Zn deficiency reduced systemic leptin levels. Changes in leptin signaling may be quite relevant to our study as leptin signaling affects mammary gland development, milk protein expression and involution [64,65]. Further studies are needed to understand if alterations in leptin signaling underlie the developmental defects we detected. In addition, Zn deficiency in male rats reduces serum testosterone and progesterone [66], suggesting changes in reproductive hormones in our model were possible and may play a role in our observations. In fact, stimulation of ER α signaling through enhanced ER α expression in the mammary gland may be at least partially responsible for these defects. ER α signaling drives ductal elongation during puberty. ER α expression is normally very low in adult mice, thus increased ER α expression indicates hormonal dysregulation in the mammary gland microenvironment. This is consistent with a previous study that showed that Zn deficiency increases ER α expression in liver [67]. Importantly, a hallmark of ER α over-expression in transgenic mice is ductal hyperplasia [68] and studies in MCF10A cells grown in culture find that estrogen treatment disrupts acini morphology and lumen formation.

The mechanisms responsible for driving ER α over-expression are not understood. However, estrogen and expression of ER α are inversely related to regulate estrogenic activity [67], suggesting that Zn deficiency may have decreased estrogen, either systemically or in the microenvironment. This finding is particularly intriguing as molecular changes in the hormonal environment in ER α over-expressing transgenic mice that give rise to precursor lesions such as hyperplasia is associated with breast cancer [68].

5. Conclusions

In summary, our study is the first to show that marginal Zn deficiency results in oxidative stress, inflammation and Zn hyperaccumulation in the mammary gland which is associated with collagen deposition and impaired mammary gland development. Importantly, hallmarks associated with increased risk of breast disease were noted. Further studies are required to understand if inadequate Zn intake in young women impairs breast development, compromise breast function during lactation or increases the risk for breast disease and cancer.

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Highlights

- In marginally Zn deficient (ZD) mice, mammary glands accumulate Zn.
- Zn accumulation is associated with changes in mammary gland microenvironment.
- ZD glands have oxidative stress, macrophage infiltration and collagen deposition.
- In ZD mammary glands ZIP6, ZIP10 and ZnT2 expression is increased.
- The microenvironment in ZD mammary gland is associated with impaired architecture.

Figure 1.

Oxidative stress was more pronounced in the mammary glands from mice fed a marginal Zn diet. Representative images of mammary glands from mice fed a Zn adequate (ZA) or a marginal Zn (ZD) diet. 4HNE staining (brown) was used to detect oxidized lipids in the mammary glands of mice fed a ZA (A, negative control; B, 4HNE) or ZD (C, negative control; D, 4HNE) diet. Magnification, 40×. Scale bar= 100 μm. As a second marker, 8-OHDG (green) was used to detect oxidized DNA in the mammary glands of mice fed ZA (J) or ZD (L). DAPI was used to stain nuclei (E, G, I, K). Mammary gland sections from mice treated orally once/wk for 4 wks with corn oil or a chemical carcinogen (DMBA, 7,12-dimethylbenz(a)anthracene) that increases oxidative stress were used as negative (F) and positive (H) controls, respectively.. Magnification, 10×. Scale bar = 200 μm. Trichrome staining (blue) was used to detect the presence of collagen in the mammary glands of mice fed a ZA (M) and a ZD diet (N). Magnification, 10×. Scale bar = 500 μm.

Figure 2.

ZIP6, ZIP10 ZnT2 abundance was higher in mice fed a marginal Zn diet. Representative immunoblots of ZIP6 (A), ZIP10 (B) and ZnT2 (C) abundance in the mammary glands of mice fed a Zn adequate (ZA, n=2) and Zn deficient (ZD, n=2) diet. Data represent mean Zn transporter abundance relative to β-actin ± SD, n=5 mice/group. Asterisk denotes significance, $p < 0.05$ and the experiment was repeated twice.

Figure 3. Mammary gland expansion and development was significantly compromised in mice fed a ZD diet. Representative images of mammary gland whole mounts from mice fed a Zn adequate (ZA, A) or a Zn deficient (ZD, B) diet. Magnification, 0.5X Arrowhead indicates terminal end structures. Scale bar 500 μ m

Figure 4.

MMP2 activity was significantly lower in mice fed a marginal Zn diet. (A) Representative image of gel zymography assay illustrating gelatinase activity at ~66 kDa (cleared band) in mammary glands from mice fed a Zn adequate (ZA, n=3) or Zn deficient (ZD, n=3) diet. Data represent mean signal intensity $\pm$ SD, n= 3 mice/group. Asterisk denotes significance, $p < 0.05$ and the experiment was repeated twice.

Figure 5.

Mammary gland morphology was substantially compromised in mice fed a marginal Zn diet. Representative images of mammary glands stained with hematoxylin and eosin. Compared with mice fed a Zn adequate (ZA) diet (A), mice fed a Zn deficient (ZD) diet (B) had extensive cellular and acellular tissue accumulation in the mammary gland. ; Magnification 10 $\times$. Scale bar=200 μ m. Compared with mice fed a ZA diet (C), mice fed a ZD diet (D) had less patent lumens. Magnification, 100 $\times$, oil. Scale bar=100 μ m. Representative images of mammary glands immunostained for e-cadherin (E, F). Compared with mice fed a ZA diet (E), mice fed a ZD diet (F) had disorganized ducts. Magnification, 100 $\times$, oil. Scale bar=100 μ m.

Figure 6.

αSMA and ERα expression was higher in mice fed a marginal Zn diet. (A) Representative immunoblots of αSMA abundance in the mammary glands of mice fed a Zn adequate (ZA, n=2) and a Zn deficient (ZD, n=2) diet. Data represent mean αSMA abundance relative to β-actin ± SD. Asterisk denotes significance, $p < 0.05$ and the experiment was repeated twice (n=5 mice/group). (B) Representative immunoblots of ERα in the mammary gland of ZA (n=2) and ZD (n=2) mice. Data represent mean ERα abundance relative to β-actin ± SD. Asterisk denotes significance, $p < 0.05$ and the experiment was repeated twice (n= 3; ZA and n=5; ZD).