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Approximately 12% of Americans do not consume the amount of zinc equal to the Estimated Average Requirement for zinc and could be at risk for marginal zinc deficiency. Zinc is an essential component of proteins in the defense against oxidative stress and DNA damage repair. Specifically in the prostate, zinc concentrations are inversely associated with prostate malignancy. Zinc deficiency may predispose cells to the development of cancer by increasing oxidative stress and DNA damage. Studies have shown that severe zinc-depletion increased DNA damage in testes. However, the effects of marginal zinc deficiency, which is more prevalent in human population and physiologically relevant, are understudied. This study aimed to specify the mechanisms by which zinc status affects DNA integrity and the prostate maintains zinc level *in vivo*. We examined the effects of zinc deficiency on DNA damage and oxidative stress in rat models and in human studies.

In rats, severe zinc-depletion caused an increase in DNA damage in peripheral blood cells that decreased following zinc-repletion. DNA repair functions were impaired as indicated by compromised p53 DNA binding and differential activation of DNA repair proteins. Importantly, marginal zinc-depletion (MZD) also increased DNA damage and oxidative stress, and impaired DNA repair functions. However, these effects were not observed in the prostate. Only in combination with an exogenous stress (exercise), MZD increased DNA damage in the prostate, indicating that MZD may sensitize the prostate to exogenous DNA damaging agents.

Similar to the rat study, marginal dietary zinc depletion (6wk) in healthy males increased DNA strand breaks in peripheral blood cells, alterations which were ameliorated by zinc repletion (4wk). Oxidative stress and antioxidants were not altered during zinc depletion/repletion periods.

The increases in DNA damage were associated with impaired zinc homeostasis. MZD decreased zinc concentration as well as ZnT2 expression in the prostate dorsolateral lobe, indicating disregulation of zinc transporter and zinc homeostasis.

Taken together, these studies suggest a key function of zinc in maintaining DNA integrity. Thus, the maintenance of adequate dietary zinc may have an important impact on protecting tissues, such as the prostate, from DNA damage and decreasing cancer risk.

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The Function of Zinc in the Maintenance of DNA Integrity *in vivo*

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Chair of the Department of Nutrition and Exercise Sciences

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Yang Song, Author

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The Function of Zinc in the Maintenance of DNA Integrity *in vivo*

Chapter 1 Introduction

1.1 Antioxidant and redox signal functions of zinc

Zinc is present in all cells and is one of the essential micronutrients for human health. Zinc ions are bound to thousands of proteins and are essential for numerous cellular functions and physiological activities, such as growth & development, immune response, wound healing, neurological function and reproduction. Unlike other essential transition metals like iron and copper, zinc does not have redox activity and serves as a good cofactor of a large number of proteins. The primary functions of zinc in biological systems can be categorized as catalytic, structural and regulatory. For instance, zinc is essential for the catalytic activities of many enzymes and functions as an electron acceptor (1). Structurally, zinc ion is required for the formation and stabilization of the protein tertiary structure. The regulatory function of zinc is related to its ability to modulate gene expression. For example, increasing intracellular zinc levels will trigger the nuclear translocation of the metal response transcription factor (MTF), which then binds to the metal response element (MRE) to induce metallothionein gene expression (2). In addition, zinc affects cell signaling pathways and regulates cell proliferation, differentiation and death (3).

There has been a great deal of interest on the roles of zinc in antioxidant defense and oxidative stress. The putative antioxidant/redox functions of zinc are outlined in the following section.

1.1.1 Antioxidant properties of zinc

A number of animal studies have provided evidence for the antioxidant properties of zinc. These studies examined biomarkers for oxidative stress in zinc-depleted animals and found that zinc-deficient rats had increased production of free radicals (4) and oxidatively modified proteins and lipids (5-8) in various tissues. Zinc deficient animals also displayed an increased susceptibility to exogenous oxidative stress such as endotoxin exposure and hyperoxia (9, 10). These studies suggest that zinc is

required to protect macromolecules such as proteins, lipids and DNA from oxidative damage.

Several mechanisms could be involved in antioxidant function of zinc. First of all, zinc may protect protein sulfhydryl groups from oxidative modification by influencing the conformation and reducing potential of thiol groups. Since the sulfhydryl groups are required for the catalytic activities of several enzymes, zinc protects the enzyme's activity from oxidative inactivation. This protection by zinc is seen in enzymes such as 5-aminolaevulinate dehydratase (11). However, not all the thiol groups bound with zinc are protected from oxidation. For instance, the thiol groups of metallothioneins have relatively low reduction potentials (-366 mV) and are easily oxidized even bound with zinc (12). Secondly, zinc may antagonize the activity of transition metals such as iron and copper. These transition metals have variable oxidation numbers and could easily catalyze the redox reactions. For example, even a small amount of free iron or copper ions could speed up autoxidation and redox reactions (via Fenton reactions), and aggravate or help to propagate oxidative damage. Several studies show that zinc may compete with copper and iron ions and prevent transition metal mediated oxidative modifications. Zago et al. examined the antagonizing activity of zinc and observed that zinc interfered with the binding efficiency of Fe^{2+} to lipoproteins in a dose dependent manner, and inhibited Fe^{2+} mediated lipid peroxidation (13). The antagonizing activity of zinc was also examined *in vivo*. Powell and his coworkers demonstrated that zinc perfusion in the isolated heart reduced copper content in the heart and attenuated the copper-associated cardiac ischemic oxidative injury (14, 15).

The third mechanism for the antioxidant property of zinc is that zinc may reduce oxidative damage indirectly by modulating antioxidant defense including (i) enzymes which catalytically remove free radicals and reactive species, like superoxide dismutase, catalase, and glutathione peroxidase; (ii) proteins which minimize the availability of pro-oxidants, like transferrins, ceruloplasmin and metallothioneins; (iii) low-molecular-mass ROS and RNS scavengers, like glutathione, ascorbic acid, uric acid and alpha-tocopherol. It has been shown that dietary zinc depletion may affect the activity or expression of these antioxidant defenses, and thereby alter susceptibility to oxidative stress.

Antioxidant enzymes such as CuZn superoxide dismutase (CuZnSOD), glutathione peroxidase (GPX) and catalase are located in different cellular compartments and have different functions. The vast majority of CuZnSOD (a zinc containing antioxidant enzyme) is in the cytosol and accelerates the dismutation of superoxide (O_2 ▪ -). Catalase in the peroxisome catalyzes the decomposition of hydrogen peroxide (H_2O_2), and catalase in erythrocytes helps to remove H2O2 diffused into erythrocytes. Glutathione peroxidase (GPX) is also responsible for the removal of H_2O_2. GPX is found mainly in the cytosol and the matrix of mitochondria, and uses GSH as its hydrogen donor. CuZnSOD, catalase and GPX act coordinately to remove free radicals and decrease oxidative stress. Mice defective in expressing antioxidant enzymes develop oxidative stress associated diseases. For instance, mice defective in CuZnSOD develop neurological damage and cancer at an accelerated rate as they age (16). GPX-1 knock out mice are much more sensitive to paraquat toxicity than the wide type mice (17).

The effects of zinc depletion on antioxidant enzymes have been examined in a number of studies (**Table 1.1**). These rat studies suggest that both severe and marginal zinc deficiencies have no effect on GPX and catalase activity, but alter CuZnSOD activity in an organ-specific manner. One human study done in a European population observed that the erythrocyte SOD activities were negatively associated with the plasma zinc concentrations, and positively associated with age. They also observed that the plasma catalase and GPX activities were similar among groups having different plasma zinc concentrations (18).

Zinc depletion affects the expression of metallothioneins. Metallothioneins are low-molecular-weight proteins with high cysteine content and high affinity for metal ions (19). They are part of the antioxidant defense, and function in restricting copper-mediated free radical reactions by scavenging singlet O_2 and OH• radicals. It has been shown that zinc regulates the expression of metallothionein *in vivo*. For example, zinc supplementation induces metallothionein mRNA or protein expression in the liver (20) and the peripheral blood cells (21), and zinc depletion increases metallothionein expression in the small intestine (22, 23) and decreases the expression in the liver or esophagus (20, 22). These decreases in metallothionein expression with zinc depletion may sensitize cells to oxidative damage.

Low-molecular-mass scavengers such as α-tocopherol, ascorbate and uric acid were also examined in a few zinc depletion studies. These studies found that zinc depletion decreased α-tocopherol and ascorbate levels in liver and lung (10), but did not change α-tocopherol level in testes (24). Moreover, plasma uric acid concentration was elevated occasionally in zinc-deficient rats (25).

1.1.2 Redox signal functions of zinc

The binding and release of zinc from ligands may serve as an important redox signal for the regulation of cell activities and physiological functions. The redox regulation is defined as using redox active species as messengers to regulate cell behavior (26). Redox messengers could be oxidative stressors (e.g. ultraviolet radiation), intrinsic redox species (e.g. glutathione/oxidized glutathione GSH/GSSG), or extrinsic redox species (e.g. ascorbic acid). These redox messengers may regulate the activities of numerous redox-sensitive signaling molecules and transcription factors by modulating intracellular redox status. The intracellular redox status is determined by the oxido-reductive status of essential thiol groups, and those thiol groups could be involved in protein phosphorylation and cofactor binding of molecules, such as zinc. McEligot AJ et al. reviewed the mechanisms by which redox status influences cellular activities including cell proliferation and programmed cell death, and proposed the involvement of redox sensitive cell signaling molecules and transcription factors(**Figure 1.1** from review (26)). Alterations in zinc status may also act as an extrinsic factor that modulates these redox sensitive signals.

Zinc can be bound with nitrogen, oxygen or sulfur. Zinc-sulfur is the most abundant form of zinc-ligand found in proteins. Both the oxido-reductive environment and the reducing potential of the thiol ligands affect the zinc-sulfur interaction. For example, the oxidation of the sulfur ligand releases zinc, and reduction restores its zinc binding ability **(Figure 1.2)** (27). This switch of zinc between the binding and release from thiol groups may change the conformation of zinc-containing proteins and modify functional activities such as DNA binding activity mediated by zinc-fingers, catalytic activity of metalloenzymes, and the nuclear translocation of transcription factors (28). Zinc may also modulate the oxido-reductive environment by changing

GSH/GSSG ratio. GSH is the predominant low-molecular-mass intracellular thiol, and GSH/GSSG ratio is one of the major indicators of redox status in cells (26). GSH/GSSG ratio between 30:1 and 100:1 helps to buffer oxidative stress generated in cell metabolism and help maintain a reductive intracellular environment. However, zinc depletion in rats decreases GSH/GSSH ratio and may increase the cell's susceptibility to oxidative stress (29-31).

These interactions among intracellular available zinc, oxido-reductive environment and zinc switches among zinc-sulfur ligands suggest the function of zinc as a redox signal in the regulation of redox-sensitive cell signaling molecules, transcription factors and enzymes, and modulation of various cell activities such as cell growth and survival, inflammation and immune responses.

1.2 Human zinc deficiency and cancer

1.2.1 Prevalence of human zinc deficiency

Human zinc deficiency was first described in Iran in the early 1960s by Prasad et al. (32, 33). They observed that zinc-deficient patients displayed symptoms of 'severe growth retardation, anemia, hypogonadism, hepatosplenomegaly, rough and dry skin, mental lethargy and geophagia' (33). Human zinc deficiency can be categorized as marginal or severe zinc deficiency, based on the severity of the symptoms. Severe zinc deficiency is rare and is predominantly found in developed countries. There also exist rare genetic inborn defects of zinc absorption, or secondary factors such as liver disease, chronic renal disease, sickle cell disease or high intakes of unleavened flour (high phytate content) that may cause more severe zinc deficiency. The affected patients suffer from severe dysfunctions of central nervous, immune, reproductive, epidermal and skeletal systems. Unlike severe zinc deficiency, marginal zinc deficiency is more common and physiologically relevant to the human zinc deficiency in developed countries such as the United States. Marginally zinc deficient patients display numerous non-specific symptoms such as growth retardation, skin changes, increased vulnerability to infection, and delayed wound healing.

According to the estimates from the International Zinc Nutrition Consultative Group (IZiNCG), approximately 2 billion people worldwide are at risk of zinc

deficiency (34). Although the true prevalence of zinc deficiency is difficult to evaluate due to the lack of sensitive and specific biomarkers (35), the actual prevalence is confirmed by the zinc supplementation trials in infants, toddlers and preschool children. These trials observed improved neurophysiologic performance, positive growth response, and significantly reduced mortality and morbidity with zinc supplementation in these populations (36-38). Although the prevalence of zinc deficiency is higher in the developing counties because of the limited availability of zinc-abundant animal sources of food such as red meat and seafood, the prevalence of marginal zinc deficiency in developed countries may still be high (39). Data from National Health and Nutrition Examination Survey (NHANES 2001-2002) supports the same conclusion; approximately 12% population do not consume the amount equals to the Estimated Average Requirement (EAR) for zinc and could be at risk of marginal zinc deficiency (13). Children, women and elderly people are at high risk of marginal zinc deficiency because of their high needs for zinc or decreased capacity of zinc absorption (40).

The role of zinc in successful aging has been recognized recently. In the elderly, inadequate dietary zinc intakes result in dysregulation of physiological functions such as immune responses, and may contribute to the development of several age-related degenerative diseases including cancer, atherosclerosis and infectious diseases (41). Studies on zinc and aging have largely focused on the effects of zinc on immune/inflammation responses (42, 43). It has been suggested that the bioavailability of zinc ion regulates the expression of pro-inflammatory cytokines and heat shock proteins such as IL-6, TNF-α and Hsp70 (44, 45), and affects TH1/TH2 balance (46). The depleted zinc status in the elderly could be both due to lowered zinc intake which is associated decreased energy demands in older individuals and impaired zinc absorption with age. However, little is known about the alterations in zinc metabolism and homeostasis during aging; differential expression of the zinc transporters in the intestinal lumen might regulate the zinc absorption ability during aging (47). Coneyworth et al suggested that methylation of *ZnT5* promoters might modulate dietary zinc absorption and contribute to the declined zinc status in elderly people (48). Understanding the mechanisms by which elderly people absorb zinc and regulate zinc homeostasis will help achieve successful aging.

Although marginal zinc deficiency is a public health problem in America, the effects of marginal zinc deficiency on human health are still unclear and understudied. Future studies focused on marginal zinc deficiency, especially in the populations at high risk, like the elderly population, will provide evidence for designing intervention strategies to decrease the prevalence of marginal zinc deficiency in humans.

1.2.2 Zinc deficiency as a risk factor of cancer

The connection between zinc deficiency and cancer has been investigated in several large observational cohort studies (49-51). These studies measured circulating zinc concentrations or evaluated dietary zinc intake of subjects, and examined the correlations between zinc status and cancer incidence. The Paris Prospective Study 2 found that male subjects aged 30-60 years with a combination of low plasma zinc and high plasma copper had an increase in all cause and cancer mortality (49). One sub-study of NHANES II found that low serum zinc concentrations were also associated with high cancer mortality (50). Another population-based follow-up study in Japan detected a negative correlation between all cause mortality and the serum Zn/Cu ratio (51). These studies suggest that zinc deficiency may increase one's risk for cancer.

The effects of zinc deficiency on esophageal cancer have been thoroughly studied by Fong et al (52-54). They used an esophageal tumor rat model that the tumor was induced by intragastric dose of N-nitrosomethylbenzylamine (NMBA). After rats were fed zinc-deficient diets for 8-14 weeks, they found that the tumor incidence and cell proliferation following NMBA treatment were increased in the zinc deficient rats in comparison with the zinc adequate rats, and zinc repletion in the zinc deficient rats increased cell apoptosis and decreased tumorigenesis (55). These results indicate that zinc depletion increases cell susceptibility to tumorigenic agents and facilitates esophageal cancer initiation and development. One possible mechanism is that zinc deficiency affects p53 gene expression and causes Has-*ras* and p53 tumor suppressor gene mutations (54).

1.3 Zinc deficiency and DNA integrity

DNA damage can be caused by numerous endogenous and exogenous DNA damaging agents such as ionizing radiation, ultraviolet light, reactive oxygen species

(ROS), ethidium bromide, and food carcinogens (polycyclic aromatic hydrocarbon PAH, heterocyclic amine HCA). In addition, micronutrient deficiencies such as vitamin C, vitamin E, iron and zinc, may damage DNA and cause DNA breaks or oxidative DNA modifications (56). Accumulation of DNA mutations is an essential event and important step in cancer initiation, promotion and progression. The initiated cancer cells obtain DNA damage by exposure to endogenous and exogenous DNA damaging agents. During the promotion stage, initiated cancer cells start to grow and differentiate rapidly, and accumulate DNA mutations resulting in changes of the cell behavior. During the progression stage, cancer cells start to display properties of aggressive growth and differentiation, and finally a small tumor forms on the site.

Although the evidence from epidemiologic studies suggests that zinc deficiency may increase the risk of cancer, the mechanisms are still unknown. One possibility is that zinc deficiency impairs DNA integrity and increases the cell susceptibility to cancer. There are several *in vitro* cell studies that have detected increases in DNA strand breaks with zinc depletion (57, 58). In vivo animal studies have also found that zinc depletion increases DNA strand breaks or DNA oxidative lesions in rat testes or monkey livers (59-61). However, many of these studies have only used severe zinc-depletion protocols which severely obstruct rats' growth and development and have little physiological relevance compared to marginal zinc depletion in the general population. Future studies need to focus on marginal zinc deficiency and examine its effects in both animals and humans.

The mechanisms by which zinc deficiency causes DNA damage are still unknown. Since zinc has antioxidant properties and may regulate cell signaling molecules and transcription factors, it has been proposed that both increases in oxidative stress and impairments of cell responses to DNA damage could be primary mechanisms by which zinc deficiency causes DNA damage.

1.3.1 Zinc deficiency impairs DNA integrity by increasing oxidative stress

Zinc depletion in animals increases oxidative stress and free radicals such as reactive oxygen and nitrogen species (ROS/RNS). The generated free radicals could directly damage lipids, proteins and DNA. ROS/RNS are endogenous DNA damaging

agents and may induce carcinogenesis by increasing DNA mutation rates *in vivo* (62). ROS/RNS directly modify DNA bases to generate oxidatively modified bases such as 8-hydroxy-2'-deoxyguanosine (8-OHdG). Resultant changes in the conformation of DNA secondary structure can induce inaccurate replication. ROS/RNS also impair DNA replication or repair by causing oxidative damage to DNA polymerase and DNA repair enzymes. All of these activities lead to the generation of DNA mutations directly or during DNA replication or DNA repair.

1.3.2 Zinc depletion impairs the cell responses to DNA damage

Normally, the accumulation of DNA strand breaks and DNA adducts stimulates cellular responses to repair the DNA damage or to remove the damaged cells. These cellular responses to DNA damage are triggered by multiple pathways including DNA repair pathways, initiation of cell cycle arrest and apoptosis, and the induction of multiple genes. Zinc depletion may interfere with some of these cellular responses and potentiate the accumulation of DNA damage.

First of all, zinc depletion may influence cell cycle arrest and apoptosis by regulating cell signaling pathways. For example, zinc deficiency decreases the circulating level of insulin-like growth factor-1 (IGF-1) (63) and disrupts intracellular receptor tyrosine kinases (RTKs) signaling transduction pathways (64), and thereby affecting cell proliferation and survival. Secondly, zinc deficiency may impair DNA repair functions. Many proteins involved in DNA repair pathways are zinc-containing metalloproteins, such as tumor suppressor gene p53, Xeroderma pigmentosum A (XPA), Replication Protein A (RPA), and poly ADP ribose polymerase (PARP). These proteins are involved in DNA repair pathways of damage recognition, nuclear excision repair (NER) or base excision repair (BER). Since zinc is essential for the function of some DNA repair proteins, zinc depletion may impair the activity of these proteins and interfere with the DNA repair functions.

p53 is one of the zinc-containing DNA repair proteins. It is called the "Guardian of the Genome", and plays an essential role in regulating DNA repair, cell proliferation and cell death (65). Mutations in the DNA binding domain of p53 protein are found in many tumor cells (65), and zinc is located in the DNA binding domain and is essential for the DNA binding activity of p53. p53 is consistently induced in zinc depleted cells,

and this induction could be attributed to the increased oxidative stress (3, 57, 65-69). However, the DNA binding activity of nuclear p53 is not increased with zinc deficiency (58, 70). Although zinc depletion increases the expression of p53, decreased intracellular zinc interferes with the incorporation of zinc ion to the DNA binding domain. Without its zinc ion, p53 looses the DNA binding capacity and is unable to function normally in cellular activities such as DNA repair and cell apoptosis.

Zinc deficiency appears to alter the activity of several non-zinc-containing redox-sensitive transcription factors, including NFκB and AP-1 (71, 72). These transcription factors regulate the cell responses to oxidative stress, cell survival and death. Zinc depletion decreases the DNA binding activity of NFkB (57, 73) *in vitro* and *in vivo*, but may or may not change that of AP-1 (57, 73). Thus, increased oxidative stress and altered intracellular redox status during zinc depletion may together modulate the expression and activity of transcription factors involved in critical biological events such as DNA repair, cell death and survival.

In summary, zinc depletion may directly increase the generation of DNA damage. At the same time, zinc depletion may also affect the activities of both zinc-dependent and zinc-independent proteins that are involved in DNA damage repair or cell apoptosis. Altogether, the accumulation of DNA damage may be substantially increased in zinc deficient cells, and thereby may predispose cells to the development of cancer.

1.3.3 Exercise as exogenous oxidative stress that impairs DNA integrity

A number of studies have examined the effects of exercise on DNA damage in human subjects. They found that after strenuous or exhaustive exercise, DMA damage of circulating leukocytes in rats or humans were significantly increased, such as DNA strand breaks, chromosomal alteration or oxidative DNA damage (74-77). However, the persistence of the exercise-induced DNA damage is likely to be protocol dependent. For example, Traber et al found that 50km ultramarathon increased DNA damage at midrace, but the damage was reduced back to the baseline 2 hours after the race (77). Hartmann et al detected maximum DNA damage at 72 hours after a triathlon competition, and substantially decreased DNA damage

120 hours after the competition (75). Therefore, the persistence of DNA damage could be a function of the duration of the protocol and the training levels of the subjects.

It has been well established that vigorous exercise induces oxidative stress and increases the generation of free radicals such as ROS/RNS. Exercise-induced oxidative damage in DNA, lipids and proteins has been detected *in vivo* (75, 77-80). Two mechanisms could be involved in this process. First of all, physical exercise enhances oxygen consumption which increases the generation of free oxygen species. Regular cellular activities constantly generate reactive oxygen species, and mitochondrial respiration is one of the main sources of reactive oxygen species such as hydrogen peroxide and superoxide in cells (81). During exercise, increased mitochondrial activity with enhanced oxygen consumption generates a higher amount of superoxide than does the sedentary status (82). Other subcellular sites such as sacroplasmic reticulum in muscle, transverse tubule in kidney, plasma membrane, phosphalipase A_2-dependent processes, and xanthine oxidase, are also potential endogenous sites for generation of ROS/RNS during exercise (82). Physical exercise can also generate free radicals through inflammatory processes. During vigorous or eccentric exercise, acute inflammation responses might play a role in ROS generation following muscle tissue injury (83). Muscle injury initiates a series of host defense reactions similar to those seen in acute inflammation, such as elevated circulating neutrophils and infiltration of muscle tissue by immune cells and macrophages (84). These leucocytes actively generate nitric oxide, superoxide, hypochlorous acid and hydrogen peroxide at the site of injury.

Although vigorous exercise increases oxidative stress in vivo, it has been suggested that chronic moderate exercise beneficially reduces oxidative damage. A number of studies done in animals show that chronic exercise training in rodents decreases oxidatively modified DNA, lipids and proteins in comparison with the sedentary rats (85-87). The possible mechanisms include the possibility that chronic exercise may reduce the generation of mitochondrially derived hydrogen peroxide (88, 89) and induce the expression of antioxidant enzymes (88, 90, 91). However, most of the chronic exercise training studies have focused on the muscle tissues, and little is

known about the effects of chronic exercise on other organ sites more susceptible to oxidative stress and DNA integrity, such as the prostate.

1.4 Functions of zinc in the prostate

The prostate contains the highest concentration of zinc of any soft tissue and secretes high amounts of zinc in prostatic fluid (92). Zinc concentrations in malignant prostate tissues are about 10-25% of in healthy prostates (93), suggesting that high zinc concentrations may be essential for the maintenance of prostate health (94-98). Epidemiologic studies observed an association between greater supplemental zinc intake and lower prostate cancer risk (99-101). However, the specific functions of zinc in the prostate and the mechanisms by which zinc maintains prostate health are still unclear, but several mechanisms have been proposed.

Prostate epithelial cells have characteristicly high aerobic glycolysis, low respiration rates (102-104) and high citrate secretion, and zinc may be required for these properties. Costello et al have found that zinc in the prostate epithelial cells reduces the activity of mitochondrial aconitase and inhibits the terminal oxidation in the electron transport chain. The inhibitory effect of zinc on m-aconitase may contribute to the properties of high citrate secretion and low respiration in the prostate (105, 106). Zinc depletion in the prostate may remove the inhibitory effects on citrate oxidation and terminal oxidation, and increase cellular respiration. Thus, decreases in cellular zinc levels in the prostate epithelial cell and subsequent release of aconitase activity could result in an elevated cell respiration that favors cell growth and differentiation, and enables these cells to manifest their malignant properties (97).

Zinc depletion induces apoptosis in most mammalian cells. Some of the possible mechanisms for apoptosis include defects in growth factor signaling pathways, activation of caspases and induction of the intrinsic pathway of apoptosis (3). However, zinc has opposite effect on cell growth in the prostate; zinc in the prostate induces mitochondrial apoptogenesis and reduces cell growth. Costello et al evaluated the effects of exposure to the physiological levels of zinc on mitochondrial apoptogenesis in three human cancer cell lines, PC-3, BPH and HPR-1 (107). They found that zinc only induced apoptosis in PC-3 and BPH which both maintained the

capacity of accumulating high intracellular zinc, but not in HPR-1 which lost its zinc accumulation ability. The effects of zinc on apoptosis in the prostate may be exerted through the releasing of cytochrome c from mitochondria (107).

The general functions of zinc in the defense against free radicals and maintaining DNA integrity may be also very important for prostate health. Depletion of zinc in the prostate may not only remove the antioxidant effects of zinc, but may also remove the inhibitory effects of zinc on terminal oxidation and increase the generation of free radicals. Therefore, the high zinc content may protect the prostate from oxidative stress by both suppressing the generation of free radicals and promoting the removal of free radicals. However, no *in vivo* studies to date have been performed to evaluate the effects of zinc deficiency in the prostate on oxidative stress and DNA damage.

The human prostate is composed by peripheral zone (70%), the central zone (25%) and the transition zone (5%), and zinc is not uniformly distributed in these zones. The peripheral zone secretes much higher levels of zinc and citrate than the other zones, and 80% prostate malignancies develop in the peripheral zone (108). Therefore, it has been postulated that the epithelial cells in the peripheral zone possess distinctive functional and metabolic properties which render them more susceptible to malignancy. The dorsolateral lobe of rat prostate is embryologically homologous to the peripheral zone of human prostate (109); the dorsolateral lobe also retains higher level of zinc than the ventral lobe (110). Therefore, the dorsolateral lobe and ventral lobe of the rat prostate may respond differently to zinc depletion and oxidative DNA damage. Future studies are needed to examine the possible differential zinc homeostasis and response of each prostate lobe to alterations in zinc status.

1.5 Zinc transporter regulation of zinc homeostasis

Total body zinc is ~1.5 to 2.5 g, and the highest zinc concentrations are found in bone, prostate and eye choroids. Zinc is widely distributed among thousands of proteins, and only very little exists as free zinc ion. It has been estimated that in mammalian cells, the concentration of the freely available zinc ranges from nanomolar to picomolar, about 0.001% of the total intracellular zinc (111), and there is little of stored zinc in the body and cells. Therefore, the mechanisms for a constant

supply of zinc and appropriate distribution of zinc to intracellular organelles and proteins are required.

Intracellular zinc levels are regulated by zinc homeostatic mechanisms which are controlled through a group of zinc transporter proteins that regulate uptake, efflux and compartmentalization of zinc. However, only limited knowledge is available about the distribution and regulation of these zinc transporters. Two zinc transporter gene families have been identified in mammals: the Solute Carrier Family 30A (SLC30A, ZnT) family and the Solute Carrier Family 39A (SLC39A, Zip) family of metal ion transporters. The ZnT and ZIP proteins appear to have opposing roles in zinc transportation and regulation of intracellular zinc concentrations. The ZnT transporters are responsible for lowering cytoplasmic zinc concentrations by transporting zinc out to the extracellular space and/or into intracellular organelles such as Golgi network, endosomes and secretory granules. The ZIP transporters are responsible for elevating cytoplasmic zinc concentrations via uptake zinc from extracellular space and or efflux out of intracellular organelles. The two transporter families act coordinately to maintain stable intracellular zinc concentrations and appropriate zinc distributions.

1.5.1 SLC30A (ZnT) family of zinc transporters

Nine members in the ZnT familiy have been identified in mammals, and most members of SLC30A (ZnT) family have 6 transmembrane domains with both N- and C- termini on the cytoplasmic side of the membrane. The exception is ZnT5 with 12 transmembrane domains. ZnT proteins are located both on cell plasma membrane and intracellular organelle membrane and function in lowering cytoplasmic zinc levels. Overexpression of some of the ZnT members render the cell resistant to zinc toxicity, indicating their role in intracellular zinc homeostasis and cell survival (112).

ZnT1 (SLC30A1) is the only zinc efflux protein found on the plasma membrane, and is responsible for exporting zinc out of the cell. Quantitative RT-PCR and Northern Blot analysis show that *ZnT1* mRNA is highly expressed in organs involved in zinc absorption, distribution and excretion such as the small intestine, kidney and placenta (112). The expression of ZnT1 can be regulated by dietary zinc intake.

Studies done in rodents have shown that zinc supplementation induces *ZnT1* mRNA and protein expression in the intestine and kidney but not liver. Dietary zinc depletion reduces *ZnT1* expression in the liver, kidney and intestine (113). However, the mechanisms by which zinc intake regulates *ZnT1* expression are still unclear. One proposed mechanism is that the increased zinc levels triggers the nuclear translocation of Metal Responsive Transcription Factor (MTF) which activates *ZnT1* transcription through binding to the *ZnT1* upstream Metal Responsive Element (MRE) (114).

ZnT2 (SLC30A2) is located on the membrane of intracellular vesicles, and functions in transporting zinc into endosome or lysosomes (115, 116). ZnT2 is not as widely distributed as ZnT1. In the rodent, *ZnT2* mRNA is detected in the small intestine, kidney and placenta (113), and the expression is regulated by dietary zinc intake. Zinc depletion reduces the *ZnT2* mRNA to an undetectable level in the kidney and small intestine, and zinc supplementation induces *ZnT2* mRNA expression in those same organs(112). High *ZnT2* mRNA levels are also detected in the prostate lateral and dorsal lobes of rats (110). Because ZnT2 is only expressed in limited number of cell types and is highly responsive to zinc intakes, it has been suggested that ZnT2 only functions when there is rapid increase of intracellular zinc (117). Kelleher et al identified a mis-sense (A->G) mutation in ZnT2 in women producing low-zinc breast milk, and the infants breastfed exclusively by these women developed zinc deficiency symptoms. They also showed that HEK-293 cells transfected with plasmid containing mutated *ZnT2* had reduced zinc secretion (116). These results show that ZnT2 plays an important role in human mammary secretary epithelial cells secreting zinc into breast milk.

ZnT3 (SLC30A3) has been detected in the brain and testis (118), and may play a role in zinc uptake into the synaptic vesicles in the pre-synaptic terminals of nerves (119). Although *ZnT3* knockout mice completely lose the ability to take up zinc in the synaptic neurons, no behavior and neurological abnormalities were observed in these knockout mice (120), and the roles of ZnT3 in neuron cells are still unclear.

ZnT4 (SLC30A4) is widely distributed in rodents, and is highly expressed in mammary glands, small intestines and the brain. ZnT4 is located on intracellular vesicles, and functions in transporting zinc into the secretory vesicles (117). One

important finding about ZnT4 is that the lethal milk disorder found in mice is caused by a mutation in *ZnT4* (121). The affected mice are unable to secrete zinc into their milk, and their pups die from zinc deficiency soon after birth**.** Although this mutation of ZnT4 has not been detected in humans, the disease identified in mice indicates that ZnT4 may have a similar function as ZnT2 of secreting zinc into milk.

ZnT5 (SlC30A5) mRNA is highly expressed in the human pancreas, ovary, prostate and testis (122), and may play roles in osteoblast maturation. Inoue et al. found that ZnT5-null mice exhibited poor growth and osteopenia, which is due to a defect in osteoblast maturation. Additionally, more than 60% male ZnT5 null mice experienced fatal arrhythmias (122)**.**

ZnT6 (SlC30A6) mRNA has been detected in the mouse liver, kidney, brain and intestine (123). Immunohistologic staining locates ZnT6 as trafficking from the Golgi network to the cell periphery (123), indicating that ZnT6 may be responsible for transporting zinc from the cytoplasm to the golgi network (123).

ZnT7 (SlC30A7) mRNA has been detected in the mouse liver, kidney, spleen, heart, brain, small intestine and lung (124), and is localized to the Golgi apparatus and intracellular vesicles in the perinuclear area (124).

1.5.2 SLC39A (Zip) family of zinc transporters

Zip stands for Zrt-, Irt-like proteins. Most of the Zip transporters have eight transmembrane domains (TMDs) with both N- and C- termini on the extracellular side of the membrane. *Zip1, Zip2, Zip3* and *Zip4* are the most extensively studied Zip members in the mammals.

Zip1 (SLC39A1) transporter is localized in the plasma membrane and endoplasmic reticulum in humans, and is widely expressed in various tissues and glands. Cells overexpressing hZip1 have dramatically increased ^{65}Zn uptake (125, 126). Reagents stimulating Zip1 mRNA degradation decreased ^{65}Zn uptake in both K562 and PC3 cells (126). These observations suggest that the ZIP1 transporter is the one of the major zinc uptake transporters in cells.

Zip2 (SLC39A2) mRNA is only expressed in low levels in a few tissues (122), and both zinc depletion and supplementation change the Zip2 mRNA expression

dramatically: Zip2 mRNA is upregulated after zinc depletion (127), and downregulated after zinc supplementation (128). Therefore, ZnT2 may play an assistant role in zinc uptake in cells. It may function in maintaining intracellular zinc homeostasis largely when dietary zinc intakes fluctuate.

Zip3 (SLC39A3) mRNA has been detected in the bone marrow, spleens, small intestines and livers of mice. The specificity of mouse Zip3 transporter for zinc has been postulated to be less than the other Zip transporters (129).

Zip4 (SLC39A4) is responsible for zinc uptake in cells and functions in zinc absorption in humans. Mutations of Zip4 gene have been identified as the cause of human acrodermatitis enteropathica, an autosomal recessively inherited disease (130-132). The affected patients are usually infants and display symptoms of severe zinc deficiency (111). The *Zip4* knockout mouse embryos die during the early stage of development, and the heterozygous offsprings have serious developmental defects and are extremely sensitive to dietary zinc depletion (133). The expression of Zip4 could be regulated by dietary zinc intakes. Andrews GK et al found that zinc depletion in dams resulted in increased Zip4 mRNA and protein expression in the intestines from the nursing neonate mice and the removal of Zip5 protein from the basolateral membrane of the enterocyte (134). Cousin RJ et al also detected markedly increased Zip4 mRNA in the small intestine of zinc-deficient mice, and the increased Zip4 proteins localized at the apical membrane of enterocyte (135).

Other Zip transporters are not studied as widely as the first four Zip members, and only a few studies examined the connection with diseases. Kagara et al reported that *ZIP10* mRNA expression in human breast cancer tissues were associated with the cancer metastasis, and knock down *ZIP10* by small interference RNA inhibited the invasive behavior of breast cancer cell MDA-MB-231 and MDA-MB-435S *in vitro* (136). A recent study done by Knoell et al have established a connection between Zip8 and inflammatory responses in lung epithelia (137). They found that Zip8 is the only zinc transporter induced by TNF-α in human epithelia *in vitro*, and knock down *Zip8* by siRNA increased the cytotoxicity to TNF-α induced inflammatory stress, and decreased cell viability.

1.5.3 Coordinated regulation of zinc transporter and diseases

Zinc homeostasis is likely maintained by the activities of a group of zinc transporters in the cell plasma membrane and intracellular organelles. At least nine ZnT and fourteen Zip family members have been identified in mammals, and their tissue expression, cellular localization and regulation are very different. The existence of this relatively large group of zinc transporters indicates that zinc homeostasis, distribution and intracellular compartmentalization are very important for regular cellular activities and survival, and are maintained by complex mechanisms. Each one of zinc transporters plays a specific role in zinc homeostasis and metabolism in specific tissues. Therefore, the identification and comparison of the expression of zinc transporters among tissues and examination of their regulations under different physiological or disease conditions will help our understanding of the tissue-specific functions of zinc transporters. A number of rodent studies have examined the mRNA and protein expression profiles of zinc transporters in tissues including placenta (138), intestine (134), liver (112, 139), kidney(112), spleen (112) and mammary gland (140). These studies provided information for understanding the mechanisms of zinc metabolism, and help to identify zinc transporters that play essential roles in zinc absorption, renal excretion, liver distribution, fetus development and immune functions.

Since it has been recognized that zinc homeostasis is essential for health, one may postulate that a loss of function or dysregulation of certain zinc transporters would result in the impairment of zinc homeostasis and predispose the body to zinc-imbalance-related diseases, such as cancer, asthma, diabetes, and Alzheimer's disease (141). For example, mutations in ZIP4 causes human acrodermatitis enterpathica and mutations in ZnT4 causes mouse lethal milk syndrome. Although zinc imbalance has been proposed as a risk factor for several chronic diseases such asthma, diabetes, Alzheimer's disease (141) and cancer (56, 142), the roles of zinc transporters in the development of these diseases are still unknown and understudied. It has been proposed that SNPs in zinc transporter genes may be associated with the increased risk of certain chronic diseases. For example, one study reported the association of polymorphism in ZnT8 with type 2 diabetes (143). However, very little

knowledge has been obtained about the polymorphism of zinc transporters and the functional connection with diseases.

The regulation of zinc transporter expression could be also important for prostate health. High zinc concentrations may be essential for prostate health, and failure to maintain zinc homeostasis may increase the risk of prostate cancer. However, the mechanisms by which the prostate accumulates high zinc and regulates zinc homeostasis are still unknown. Several studies have been done to explore the link between zinc transporters and risk of prostate cancer. Zinc transporters including ZnT1, ZnT3, Zip1, Zip2 and Zip3 have been found to be associated with the low intracellular zinc content in the human prostate cancer tissues or prostate epithelial cancer cell lines. Rishi et al found that normal prostate tissues from African American men had higher expressions of *ZIP1* and *ZIP2* than the tissues from white men, which could be associated with higher incidence of prostate cancer in African Americans (94). Costello LC et al found that *hZIP1, hZIP2 and hZIP3* gene and/or protein expressions were downregulated in human prostate adenocarcinoma glands and malignant cell lines (98). Huang LP et al found that the tumorigenic human prostate epithelial cell line RWPE2 had decreased ZIP1 protein expression and redistributed intracellular ZIP3 in comparison with the non-tumorigenic human prostate epithelial cell line RWPE1 (95). Overexpression of *hZIP1* in the tumorigenic prostate cell also increased the intracellular zinc concentrations, induced cell apoptosis and suppressed cell growth (95). These results suggest that lack of regulation in ZIP1 expression or ZIP3 localization may predispose prostate cells to cancer development, and restore the expression of ZIP may suppress the malignant transformation of prostate cells. For the ZnT family members, Yamanaka et al detected lower expression of ZnT1 gene in human prostate cancer tissues (144) and Hirano et al found the ZnT1 gene expression was associated with the low intracellular zinc content in the androgen-independent subline of LNCaP (145). Beck et al also detected lower levels of ZnT4 gene expression in human prostate benign hyperplasia (BPH) and carcinoma tissues (146). In summary, a number of zinc transporters have altered expression or localization in the prostate malignant tissues. This may contribute to the prostate cancer initiation, promotion and progression.

1.6 Dissertation specific aims and hypothesis

Approximately 12% of Americans do not consume an amount of zinc equal to the Estimated Average Requirement (EAR) for zinc and could be at risk of zinc deficiency. One important consequence of zinc deficiency is an increased susceptibility to oxidative stress. Oxidative stress makes a significant contribution to the pathology of several human diseases, such as cancer, diabetes, and Amyotrophic Lateral Sclerosis (ALS). In particular, cancer is the one of the leading causes of death in America. The association between zinc deficiency and cancer has been suggested by several large observational studies where low serum zinc concentrations were associated with increased risk of cancer mortality. Specifically in the prostate, zinc concentrations are inversely associated with prostate malignancy; when normal prostate develops into cancer and to the late stages of androgen-independent cancer, zinc concentrations in the prostate are continuously decreased. Consequently, zinc may play a key role in maintaining prostate health.

The overall goals of this study are to identify the roles of zinc in maintaining cellular or tissue health and preventing cancer development. The objectives of this study are to specify the mechanisms by which zinc status may alters DNA integrity and oxidative stress, and the mechanisms by which prostate maintains zinc level *in vivo*. We hypothesize that dietary zinc levels will affect DNA integrity through modulating oxidative stress and antioxidant defense, and regulating DNA repair pathways *in vivo*. We also hypothesize that dietary zinc levels will alter intracellular zinc content and the regulation of zinc homeostasis in the prostate. The rationale behind our hypothesis is that zinc displays antioxidant properties *in vitro* and *in vivo*. Zinc depletion in cells including prostate epithelial cells increases DNA strand breaks, and zinc deficient rats have increased susceptibility to exogenous oxidative stress and increased generation of oxidatively modified lipids and proteins. To test our hypotheses, we will accomplish the following specific aims:

Aim 1: Identify the effect of zinc depletion in rats on DNA integrity, oxidative stress and the susceptibility to exogenous oxidative stresses in peripheral blood and/or the prostate *in vivo*.

Our working hypothesis is that zinc depletion will impair DNA integrity and increase rats' susceptibility to exogenous stress such as chronic exercise by increasing oxidative stress and interfering with antioxidant defense and DNA repair pathways *in vivo*, and zinc repletion will revere these deleterious effects. To test this hypothesis, we will examine the effects of dietary severe zinc depletion/repletion and marginal zinc depletion in rats on DNA damage, oxidative stress biomarkers, antioxidants and DNA repair proteins in peripheral blood cells and the prostate. We will also examine these parameters in chronic exercising rats, as an exogenous oxidative stress with marginal zinc depletion

Aim 2: Identify the effects of zinc depletion and repletion on DNA integrity and oxidative stress in humans.

Our working hypothesis is that dietary zinc depletion and repletion will alter DNA integrity, oxidative stress and antioxidant defense in humans. To test this hypothesis, we will use a dietary zinc depletion and repletion protocol in humans to examine DNA damage in peripheral blood cells, and oxidative stress biomarkers and antioxidants in plasma and erythrocytes.

Aim 3: Specify the mechanism by which prostate maintains zinc homeostasis.

Our working hypothesis is that dietary zinc fluctuation will affect zinc homeostasis by regulating zinc transporter expressions in the prostate. We will examine zinc transporter expression profile in the prostate and identify the transporters which are important for prostate zinc homeostasis and prostate health.

Table 1.1 Zinc deficiency affects the activity of antioxidant enzymes in different tissues

		Liver	**Lung**	**Intestine**	**Blood**
CuZn	(147)	↓			
SOD	(148)*	↓			
	(149)*	↓			↑
	(20)	↓			
	(150)	↔	↑		
	(151)^		↑		
	(8)			↑	
GPX	(147)	↔			
	(150)	↔	↔		
	(151)^		↔		
Catalase	(150)	↔	↔		
	(151)^		↔		

* no pair-fed group included
^ used marginal zinc depletion protocols

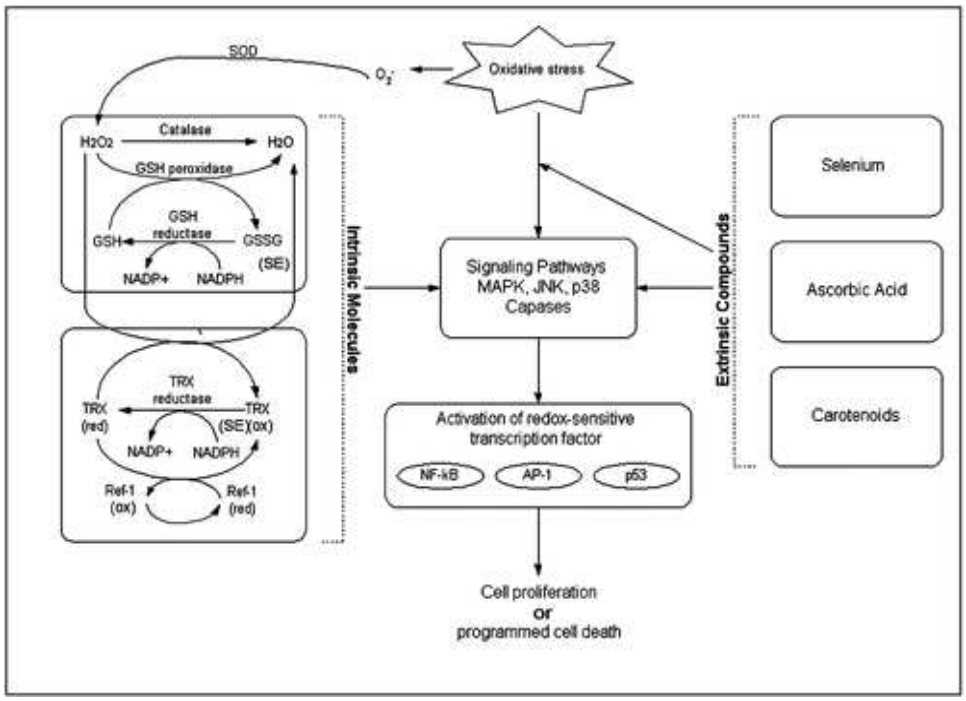

Figure 1.1 Oxidative stress, intrinsic molecules and extrinsic molecules modify the redox status of cell. The modified signaling molecules and transcription factors change the cell biological processes such as cell proliferation or programmed cell death. This figure is adapted from paper published by McEligot AJ et al. (27).

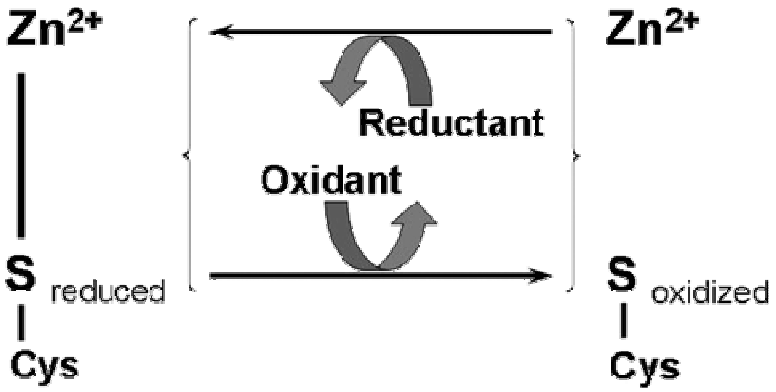

Figure 1.2 Redox regulated Zn-sulfur interaction. The oxidation and reduction of the sulfur ligand are coupled with the binding and release of zinc, thus generating redox zinc switches in proteins. This figure is adapted from paper published by Maret W. et al.(27)

Chapter 2 Zinc deficiency affects DNA damage, oxidative stress, antioxidant defenses and DNA repair in rats

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2.1 Abstract

Approximately 12% of Americans do not consume the Estimated Average Requirement for zinc and could be at risk for marginal zinc deficiency. Zinc is an essential component of numerous proteins involved in the defense against oxidative stress and DNA damage repair. Studies *in vitro* have shown that zinc depletion causes DNA damage. Therefore, we hypothesized that zinc deficiency *in vivo* would cause DNA damage through increases in oxidative stress and impairments in DNA repair. Sprague-Dawley rats were fed zinc-adequate (30 mg Zn/kg), severely zinc-deficient (<1 mg Zn/kg), or pair-fed diets for 3 wk. After zinc depletion, rats were repleted with zinc-adequate diet for 10 d. In addition, zinc-adequate (30 mg Zn/kg) or marginally zinc-deficient (6 mg Zn/kg) diets were given to a different group of rats for 6 wk. Severe zinc deficiency caused a significant increase in DNA damage in peripheral blood cells that decreased back to control following zinc repletion. We also detected impairments in DNA repair functions as indicated by compromised p53 DNA binding and differential activation of base excision repair (BER) proteins OGG1 and PARP. Importantly, marginally zinc-deficient rats also had significant increases in DNA damage and plasma F_2-isoprostanes, and impairments in DNA repair functions. However, plasma antioxidant capacity and erythrocyte SOD activity were not affected by either marginal or severe zinc deficiencies. These results suggest interactions among zinc deficiency, DNA integrity, oxidative stress and DNA repair, and implicate the role of zinc in maintaining DNA integrity.

Keywords: zinc, oxidative stress, DNA integrity, DNA damage, DNA repair

2.2 Introduction

Worldwide, zinc deficiency is an important public health problem with approximately 2 billion people who do not ingest adequate amounts of zinc (34). Data from NHANES 2001-2002 show that approximately 12% Americans do not consume the Estimated Average Requirement (EAR) for zinc, thus a large proportion of U.S population could be at risk for marginal zinc deficiency (147). There is a growing body of evidence that suggests that zinc deficiency may increase the risk for cancer. Epidemiological studies reveal associations between low circulating zinc concentrations and increased risk of cancer (49, 50). However, the mechanisms by which zinc deficiency increase the risk of cancer are still unclear and understudied. Zinc is an important element in numerous transcription factors, antioxidant defense enzymes and DNA repair proteins, and plays a pivotal role in several essential cell functions such as cell proliferation and apoptosis, defense against free radicals and DNA damage repair. For instance, CuZn superoxide dismutase is an important first line defense enzyme against oxygen radical species, and p53 is an important zinc-containing transcription factor that plays an essential role in the DNA damage response. Low cellular zinc may increase oxidative stress, impair DNA binding activity of p53 and interfere with its functions in DNA repair (57, 73). Thus, several different mechanisms may be involved in processes leading to impaired DNA integrity with zinc deficiency *in vivo*: 1) zinc deficiency may increase oxidative stress that directly causes DNA damage; 2), zinc deficiency may impair DNA damage repair responses (142).

Although increasing evidence suggests that zinc has antioxidant properties and protects tissue from oxidative damage (4-10, 57, 152), many of these studies have only used severe zinc-depletion protocols that obstruct growth and development, and have little physiological relevance in the general population. In contrast, marginal zinc deficiency is more physiologically relevant to human zinc deficiency (153), yet little is known about its effect on oxidative stress and DNA damage.

Our current rat study examined the in vivo interactions among zinc deficiency, DNA damage, oxidative stress, antioxidant defenses and DNA repair in both severely and marginally zinc-depleted rats. We hypothesized that alterations in zinc status would affect DNA integrity by altering oxidative stress, antioxidant defenses and DNA

repair functions. Previous studies done in our lab have demonstrated that severe zinc depletion increases oxidative stress biomarkers in rat plasma (152). In the current study we further assessed DNA damage and DNA repair proteins in zinc-depleted rats, and importantly added a zinc-repletion stage to test whether these deleterious effects are reversible. Secondly, we used a physiologically relevant marginally zinc-depleted rat model and investigated the effects of marginal zinc-deficiency on DNA integrity, oxidative stress and DNA repair. Therefore, our study is one of the first to explore the effects of marginal zinc-deficiency on DNA integrity *in vivo*, and may shed light onto human trials exploring the possible deleterious effects of marginal zinc deficiency in humans.

2.3 Material and Methods

Rats and diets. The rat protocol was approved by Oregon State University's (Corvallis, OR) Institutional Laboratory Animal Care and Use Committee. Male Sprague-Dawley rats from Charles River (Wilmington, MA) were acclimated for 1 wk to the temperature- and humidity-controlled environment with a 12-h-dark: light cycle. The rats for the severe zinc deficiency study were maintained in stainless steel suspended cages, and the rats for the marginal zinc deficiency study were maintained in polycarbonate cages. Diets were based on modified AIN-93G rodent diets (154) for growing rats or AIN-93M diets (154) for sexually mature rats, formulated with egg white rather than casein and with zinc provided as zinc carbonate (Dyets, Bethlehem, PA). Deionized water was provided as drinking water.

Severe zinc deficiency study. Rats (10/group, 3 wk old, ~50g) were randomly assigned to three dietary treatments: zinc-adequate diet (ZA, 30 mg Zn/kg), severely zinc-deficient diet (ZD, <1 mg Zn/kg), or pair-fed zinc-adequate diet (PF, 30 mg Zn/kg) to match the mean feed intake in the ZD rats. Ten rats fed ZD diet for 21 d were switched to zinc-adequate diet for up to 10 d for the zinc repletion group (ZnRe). Diet intakes and body weights were measured daily. Rats were killed following anesthesia with isoflurane overdose (1-5%; Henry Schein; Melville, NY).

Marginal zinc deficiency study. Rats (12/group, 5 wk old, ~ 110g) were randomly assigned to one of two dietary treatments: zinc-adequate diet (MZA, 30 mg Zn/kg) or

marginally zinc-deficient diet (MZD, 6 mg Zn/kg) for 42 days. Diet intakes and body weights were measured twice every week. Rats were killed following anesthesia with isoflurane overdose (1-5%; Henry Schein; Melville, NY).

Tissue and blood collection. Blood samples were collected by cardiac puncture into trace element-free vials containing EDTA. Plasma was separated immediately and frozen at -80ºC until analysis. A portion of plasma for vitamin C analysis was acidified (1:1) with 10% perchloric acid containing 1 mmol/L DTPA (diethylenetriaminepentaacetic acid), and centrifuged (15,000 x *g*, 5 min, 4 ℃). The supernatant fraction was removed, snap frozen, and stored at -80℃ for future analysis of ascorbic acid. Erythrocytes were washed three times with cold saline solution without EDTA, and frozen at -80° for furth er analysis. Samples of liver were dissected and immediately snap frozen at -80℃ unti l analysis.

Zinc analysis. Liver zinc concentrations were determined by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES; Teledyne Leeman Labs, Hudson, New Hampshire) as described previously, with minor modifications (155). Liver samples were digested in 1mL 69%-70% OmniTrace nitric acid (VWR, West Chester, VA) overnight. Following digestion, samples were diluted 10 times with water treated with chelex 100 resin (BIO-RAD, Hercules, CA) and analyzed by ICP-OES against known standards (152).

DNA damage. Single-strand breaks in peripheral blood cells were determined by alkali single-cell gel electrophoresis (Comet assay) as described by Singh et al. (156). Comet measurements were made by image analysis using Nikon E400 fluorescent microscope and Comet Assay III software. Images of 100 randomly selected nuclei (50 nuclei from each of two replicate slides) were analyzed from each sample. The comet measurements of tail moment were recorded and used to indicate DNA damage in rat peripheral blood cells. One comet slide was made for each rat, and on each slide 50 comets were scored blindly for tail moments. Results are presented as fold of the tail moments to those of ZA or MZA group.

Oxidative stress. Plasma F_2-isoprostanes were measured as an index of lipid peroxidation and indicator of oxidative stress *in vivo*. The sum of various F_2-isoprostanes with the appropriate mass/charge ratio and fragmentation characteristics and arachidonic acid were measured in plasma as previously described (157). Briefly, plasma was subjected to alkaline hydrolysis, acidified, extracted with ethyl acetate-hexane, and dried under N_2 gas. The concentrated extract was separated by HPLC (High Performance Liquid Chromatography Shimadzu HPLC system). The prostaglandin F (PGF) analytes and arachidonic acid were detected using multiple reaction monitoring (MRM) on an Applied Biosystems/MDS Sciex API 3000 triple quadrupole mass spectrometer. Deuterated internal standards of isoprostanes (d_4-8-iso-$PGF_{2\alpha}$ and d_4-$PGF_{2\alpha}$) or arachidonic acid (d_8-arachidonic acid) were used to ensure reliable quantitative analysis.

Total antioxidant capacity. To assess plasma total antioxidant capacity, the ferritin reducing ability of plasma (FRAP) was measured at 550 nm on a microplate reader (Spectramax 190; Molecular Devices, Sunnyvale, CA) as previously described (158). FRAP values were calculated using trolox as standards. Plasma α-tocopherol and vitamin C were also measured. Plasma α-tocopherol was extracted and measured by HPLC-ECD (high performance liquid chromatography-electrochemical detection as described (159, 160). Plasma ascorbic acid were measured by HPLC-ECD as described (161).

Erythrocyte SOD activity. SOD activity was determined by the xanthine oxidase-cytochrome c method according to McCord and Fridovich (162) and L'Abbe et al (163). Hemoglobin concentration in cell lysate was determined by Drabkin's method (164). The rate of increase in absorbance at 408 nm was monitored by plate reader (Molecular Device, CA). One SOD activity unit was defined as the quantity of SOD required producing 50% inhibition of the reduction rate of cytochrome c under the experimental conditions.

Western analysis of DNA repair proteins. Protein concentrations in liver lysate were determined using *DC* Protein Assay (Bio-Rad, Hercules, CA). Proteins

(30µg/lane) were separated by SDS-PAGE on a 4-12% bis-Tris gel (Invitrogen, CA) and transferred to nitrocellulose membrane (Bio-Rad, CA). Equal protein loading was confirmed with β-actin levels. The primary antibodies used for detection were mouse anti-p53 (Ab-1, Calbiochem, CA), mouse anti-Poly ADP ribose polymerase (PARP, BD pharmingen, CA), rabbit anti- 8-oxoguanine glycosylase (OGG1, Novus Biologicals, Littleton, CO), and mouse anti-β-actin (Sigma). Bound antibodies were detected using either goat anti-mouse IgG-HRP or goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology), and developed with SuperSignal West Femto chemiluminescent substrate (Pierce, Rockford, IL). Images were acquired on Alpha Innotech photodocumentation system (Alpha Innotech, Hayward, CA) and analyzed using Image J 1.37v software (NIH, Bethesda, MD).

Electrophoretic mobility shift assay (EMSA). Nuclear protein was extracted from frozen liver using a Nulear Extract Kit (Active Motif North America, Carlsbad, CA). p53 DNA binding activity was assessed by EMSA using the p53 IRDye 700 Infrared Dye Labeled Oligonucleotides (LiCOR Biosciences) as directed by the manufacturer. Briefly, 5 μg nuclear extract, 50 fmol p53 oligo IRDye 700 Infrared Dye, 1 μg poly(2'-deoxyinosinic-2'-deoxycytidylic acid), 2 μL 10x binding buffer (1 mol/L Tris, 1 mol/L KCl, 1 mol/L dithiothreitol, and 500 mmol/L EDTA), 2 μL 25mmol/L dithiothreitol plus 2.5% Tween-20, and 2 μL loading dye was incubated at room temperature in a total reaction volume of 20 μL for 30 min. For specific competitor reactions, the sample was incubated for 10 min with 5 pmol of unlabeled p53 oligo before addition of 50 fmol of labeled probe. Reaction mixture was separated on a 6% acrylamide gel at 150 V for 2 h and then imaged and quantified using the Odyssey Infrared Imaging System.

Statistical analysis. Statistical analysis was performed with the use of PRISM (version 4.0; GraphPad Software, San Diego). One-way ANOVA were used for all comparisons between dietary treatments with Tukey's post-hoc test when appropriate. Student's t test was used for comparisons between MZD and MZA groups. Equal variances among groups were tested by Bartlette's test, and appropriate data transformation was taken to account for unequal variances. Differences were

considered statistically significant at $P<0.05$. All data are reported as mean ± SEM (standard error of the mean) unless otherwise indicated.

2.4 Results

Body and organ weights. Severe dietary zinc restriction (<1 mg Zn/kg) resulted in anorexia and lower growth rates in the ZD rats than in the ZA and PF rats (data not shown). In addition, the ZD rats displayed other signs of zinc deficiency including loss of hair, decreased activity, and increased agitation. At the end of zinc depletion, body weight in the ZD group was lower (110.8 ± 3.5 g) than in the ZA (226.8 ± 7.5 g) and PF (136.6 ± 2.3 g) groups ($P<0.05$). Liver and spleen weights in the ZD group were also significantly lower than in the ZA and PF groups ($P<0.05$, data not shown). Repletion of the ZD rats with zinc-adequate diet for 10 d partially restored body weight in the ZnRe group (202.0 ± 4.9 g), but it remained 31.7% lower than in the ZA group (291.6 ± 10.0 g, $P<0.05$). However, the growth rate (as indicated by rate of body weight gain) during zinc repletion (9.5 g/d) was 863.0% of that during zinc depletion (1.1g/d) and greater than the growth rate of the ZA group (6.6 g/d). Liver and spleen weights were also restored by zinc repletion (data not shown). Marginal zinc depletion also had no effect on body and organ weights, as expected. At the end of the study, the body weight was 360.3 ± 12.8 g in the MZA group and 380.6 ± 8.4 g in the MZD group.

Tissue zinc concentrations. Hepatic zinc concentrations were lower in the ZD rats (0.53± 0.03 μmol/g) than in the ZA rats (0.79± 0.09 μmol/g, $P<0.001$) and PF rats (0.64± 0.03 μmol/g, $P=0.07$). These data in combination with the physiological alterations observed in the ZD rats confirmed that the rats fed the severely zinc-deficient diet developed zinc deficiency. The 10-d zinc repletion increased hepatic zinc concentrations in the ZnRe rats (0.72 ± 0.03 μmol/g) to the control levels, suggesting that the rats achieved zinc-adequate status following the repletion period. The MZD rats also had significantly lower hepatic zinc concentrations (0.66 ± 0.03 μmol/g) than the MZA rats (0.75 ± 0.02 μmol/g, $P<0.05$), confirming altered zinc status in the marginal zinc depletion model.

DNA damage in peripheral blood cells. The mean tail moment of the ZD group was 1.31 fold of the PF rats **(Figure 2.1A** *P*<0.05**),** indicating an increase in single-strand breaks with severe zinc deficiency. The 10-d zinc repletion reduced the tail moment back to that of controls (Figure 2.1A, PF vs ZnRe, *P*>0.05**)**, suggesting that DNA damage was reversible with zinc repletion.

The mean tail moment of the MZD group was 1.2 fold of the MZA group (**Figure 2.1B**, *P*<0.05), indicating that marginal zinc deficiency is sufficient to increase DNA damage.

Oxidative stress in plasma. Plasma arachidonic acid (AA) concentrations, the precursor of F_2-isoprostanes, were significantly lower in the ZD rats and their PF rat controls than in the ZA rats (*P*<0.05, data not shown). When plasma F_2-isoprostane concentrations were normalized to arachidonic acid concentrations, the ZD group had more oxidative stress than the ZA group, as indicated by increases in plasma 15-series and 5-series F_2-isoprostane concentrations (**Figure 2.2A&B**, *P*<0.05). Increases in oxidative stress with zinc-deficiency were reduced by the zinc repletion to the control levels (Figure 2.2A&B). However, the plasma F_2-isoprostane concentrations in the ZD and PF groups were not different. It is likely that the severe feed restriction in itself is a marked stress on the rats that could result in high oxidative stress. Another possible explanation is that feed restriction decreased zinc intake in addition to total caloric intake in PF rats (57% of the mean zinc intake as the ZA rats), effectively causing a marginal zinc deficiency. The significant 20% decrease hepatic zinc concentrations in the PF compared to the ZA group (*P*<0.05) further supports the concept that zinc status is impaired in the PF rats.

Importantly, the MZD rats also had significantly higher plasma 15- and 5-series F_2-isoprostane concentrations (**Figure 2.2C&D**, *P*<0.01), yet plasma arachidonic acid levels remained unchanged by marginal zinc depletion.

Antioxidant defenses: FRAP, vitamin C, vitamin E and erythrocyte SOD. The ZD group had significantly lower plasma FRAP values, as well as ascorbic acid and α-tocopherol concentrations, than the ZA group (*P*<0.05) but not the PF group; dietary zinc repletion restored those to control levels (**Table 2.1**). Hepatic α-tocopherol levels

in the ZD group were significantly lower than in the ZA and PF group (Table 2.1, *P*<0.05), similar to our previous findings (152).

The MZD group had similar antioxidant status to the MZA group (**Table 2.2**), suggesting that antioxidant defenses are inadequate to prevent the oxidative stress and DNA damage observed with marginal zinc deficiency.

Erythrocyte superoxide dismutase (SOD) is comprised of cytosolic CuZnSOD, an important zinc-containing antioxidant defense enzyme in the circulation. Erythrocyte SOD activities were not altered with either severe or marginal zinc deficiencies (Table 2.1 & 2.2).

Zinc-containing DNA repair proteins in livers. OGG1 protein levels in livers were substantially higher in the ZD group than in the ZA and PF groups (**Fig 2.3A**, *P*<0.05), and were reduced back to control levels after zinc repletion. The MZD group also had significantly higher OGG1 protein levels (**Fig 2.3D**, *P*<0.05) than the MZA group. However, PARP protein levels in livers showed no statistical alterations after severe and marginal zinc depletions (**Fig 2.3B&E**).

The ZD group had significantly higher p53 protein levels in livers than the PF and ZA groups (**Fig 2.3C,** P<0.05), and zinc repletion reduced p53 protein back to control levels. However DNA binding activity of p53 in liver nuclear extract was unchanged (data not shown), suggesting the functional activity of p53 might be compromised. The MZD group did not have altered p53 protein levels in livers (**Fig 2.3F**, P>0.05), and DNA binding activity of p53 was unchanged (data not shown) despite having higher DNA damage than the MZD group. Together, DNA repair pathways may be impaired with severe and marginal zinc deficiencies.

2.5 Discussion

The present study shows that both severe and marginal zinc deficiencies *in vivo* increase oxidative stress, impair DNA integrity and increase DNA damage in rat peripheral blood cells. Concomitant with increases in DNA damage are impaired DNA repair functions with zinc deficiency. Importantly, DNA damage and oxidative stress biomarkers are reversed upon zinc repletion. This study highlights the importance of

zinc in the maintenance of DNA integrity and points out that even marginal zinc deficiencies have deleterious consequences and may increase one's risk for DNA damage.

The mechanisms by which zinc deficiency affects DNA damage are unclear. We have previously postulated that increases in DNA damage with zinc deficiency is a multi-factorial process involving both perturbations in oxidative stress and compromised DNA repair (142). Perturbations in oxidative stress with zinc deficiency may be attributed to the antioxidant functions of zinc; however, the mechanisms by which zinc protects macromolecules from oxidative modification are not completely understood. The current study shows that in the circulation alterations of antioxidant scavenger levels in plasma (FRAP, ascorbic acid and α-tocopherol) and erythrocyte SOD activity do not depend on zinc levels. However, unlike plasma α-tocopherol, hepatic α-tocopherol levels were reduced with zinc depletion. On the other hand, plasma F_2-isoprostanes levels, an index of lipid peroxidation, were the same in both the ZD rats and PF rats. It is possible that the liver compromises its own vitamin E status to maintain plasma α-tocopherol levels, and thus suppresses lipid peroxidation caused by zinc depletion. However, further studies are required to confirm the effects of zinc deficiency on the metabolism and transportation of hepatic vitamin E.

Although zinc may not regulate antioxidant defenses directly, several other mechanisms could be involved in the antioxidant function of zinc. First of all, zinc protects sulfhydryl groups in proteins from oxidation and helps maintain normal functions of proteins (11). Zinc may modulate the oxido-reductive environment in cells through modulation of thiol status. Thus, zinc depletion *in vivo* may change intracellular environment from reductive to more oxidative status (7, 29-31), and make cells vulnerable to oxidative stress. Secondly, zinc antagonizes the activities of bivalent transition metals including iron and copper, and prevents the deleterious free-radical reactions (e.g. Fenton reaction) stimulated by iron and copper. Thirdly, zinc is a component of metallothioneins which are part of classic antioxidant defenses. Metallothionein levels are decreased with zinc deficiency in liver and esophagus (20, 22). Our lab has also detected a decrease in prostate metallothionein expression with zinc deficiency *in vivo* (data not published). A lack of metallothionein may further

sensitize cells to oxidative insults. Further exploration of these potential mechanisms is an important area of future research.

Increases in DNA damage with zinc deficiency may not only be due to perturbations in oxidative stress, but also due to compromised DNA repair functions. In the current study, repletion of zinc deficiency reversed DNA damage in peripheral blood cells containing both short-lived neutrophils and long-lived lymphocytes, suggesting that DNA damage is repaired in long-lived cells and/or eliminated with damaged cells through cell death or cell apoptosis. Normally, accumulation of DNA damage stimulates cell responses, including DNA repair, cell cycle arrest and apoptosis, which help repair DNA damage and inhibit the accumulation of mutations. However, in zinc-deficient rats, DNA damage responses may be compromised, which causes the accumulation of DNA damage and the possible increases in cancer risk.

Different mechanisms could be involved in altering DNA damage responses in zinc-deficient rats. Loss of intracellular zinc may decrease the expression or impair the function of DNA repair proteins, thereby interrupt DNA repair pathways. For example, p53 is a zinc-containing protein that plays an essential role in regulating DNA repair, cell proliferation and cell death (65). Zinc is located in the DNA binding domain and is essential for the DNA binding activity of p53. p53 expression is induced in zinc depleted cells and rats (57, 58, 65). However, the current study and our previous *in vitro* studies (58, 70) show that the DNA binding activity of p53 is impaired by zinc deficiency. The current study also assessed two other DNA repair proteins, PARP and OGG1, which play pivotal roles in the BER pathway. 8-hydroxyl-2'-deoxyguanosine (8-OHdG) is a biomarker for oxidative DNA damage, and is one of the major targets of BER. OGG1 functions in the first step of BER pathway to recognize and remove 8-OHdG (165). PARP binds to DNA single-strand breaks (SSB) created by OGG1 through its zinc finger motif and recruits other DNA repair factors (eg, XRCC and DNA ligase) to the nick to complete the whole repair process (166, 167). Although both of them have zinc-finger motifs, zinc status appears to affect their expression differently. These results point out a potential hierarchy of zinc-related protein function that may be preserved in the absence of cellular zinc levels. The current study is the first to show that OGG1 expression is dramatically increased by marginal and severe zinc depletion, indicating increased oxidative DNA damage with

zinc deficiency. However, PARP capacity and expression is decreased or only slightly altered with zinc deficiency (168, 169). This lack of response or negative response of PARP to zinc depletion could markedly interrupt the overall BER pathway and contribute to the accumulation of DNA damage. Although marginal and severe zinc deficiency may affect DNA repair proteins differently, overall they both impair DNA repair functions and disable cells to get rid of oxidative DNA damage.

In the US, approximately 12% people do not consume enough zinc (147). Marginal zinc deficiency is proposed to be highly prevalent in humans with a low consumption of seafood and meat and/or high consumption of phytate. Infants, children, women and elderly people are at high risk of marginal zinc deficiency because of either high nutrient requirements or compromised digestion and absorption functions (40). The current study confirms that zinc depletion, including marginal zinc deficiency, promotes DNA damage. The impairment of DNA integrity could have important impact on several processes involved in immune function, cancer and other degenerative disorders. However, these deleterious effects of zinc deficiency on DNA integrity do appear to be reversible. The current study suggest there exist complex *in vivo* interactions among zinc deficiency, DNA integrity, oxidative stress and DNA repair, and implicate the role of zinc in maintaining DNA integrity.

Acknowledgements:

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Table 2.1 Plasma and tissue antioxidant status in rats fed a ZD, PF or ZA diet for 21 d, or in rats replete with ZA diet for another 10 d as ZnRe[1]

Antioxidant	Plasma FRAP	Plasma ascorbic acid	Plasma α-tocopherol	Hepatic α-tocopherol	Erythrocyte SOD activity
	μmol/L		*nmol/g*		*U/mg Hb*
ZA	247.0±21.8[a]	79.3 ± 7.8[a]	18.8 ± 1.0[a]	127.1 ± 12.2[a]	100.1 ± 4.2[a]
ZD	169.2 ± 5.8[b]	31.4 ± 2.8[b]	13.0 ± 0.9[b]	58.1 ± 6.9[c]	101.7 ± 4.1[a]
PF	160.9 ± 5.1[b]	28.3 ± 3.7[b]	14.0 ± 0.9[b]	90.8 ± 9.8[b]	105.1 ± 4.2[a]
ZnRe	258.9 ± 9.6[a]	75.0 ± 7.0[a]	20.7 ± 0.7[a]	100.7 ± 5.6[a,b]	89.0 ± 6.6[a]
P-value	<0.0001	<0.0001	<0.0001	<0.0001	0.12

[1] Values are means ± SEM, *n*=10. Means in a column with superscripts without a common letter differ, *P*<0.05.

Table 2.2 Plasma and tissue antioxidant status in rats fed a MZD or MZA diet for 42 d[1]

Antioxidant	Plasma FRAP	Plasma ascorbic acid	Plasma α-tocopherol	Hepatic α-tocopherol	Erythrocyte SOD activity
	μmol/L		*nmol/g*	*U/mg Hb*	
MZA	183.8 ± 4.9	50.9 ± 3.8	16.1 ± 1.6	78.2 ± 4.1	100.6 ± 3.5
MZD	176.4 ± 5.1	49.3 ± 3.0	15.7 ± 1.8	79.4 ± 5.0	100.3 ± 5.8
P-value	0.3	0.73	0.87	0.85	0.3

[1] Values are means ± SEM, n=12. Significant differences between means are determined by student's t-test.

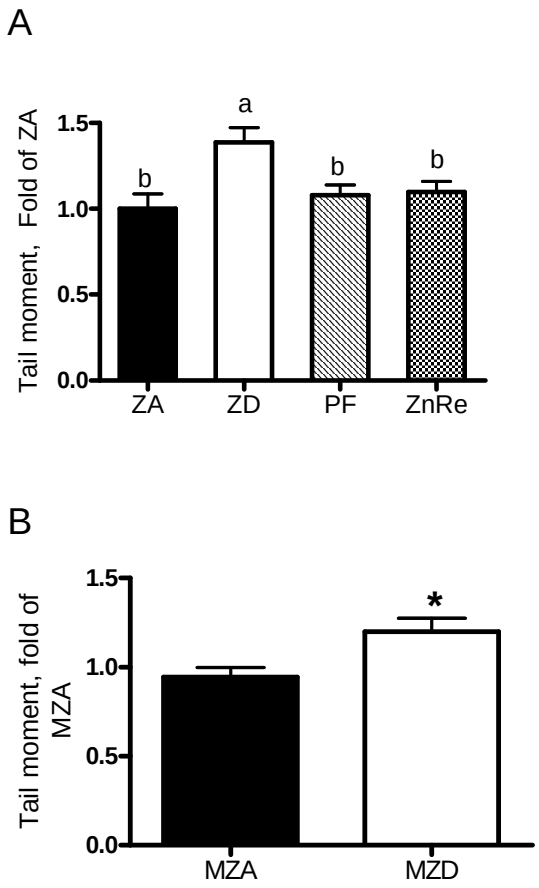

Figure 2.1 Effects of dietary zinc status on DNA single-strand breaks in rat peripheral blood cells. DNA single-strand breaks were measured by Comet assay as described in *Materials and Methods*. In (A), rats were fed a ZD PF, or ZA diet for 21 d, and then some ZD rats were switched to the ZA diet for 10 d as zinc repletion. In (B), rats were fed a MZD or MZA diet for 42 d. Values are means ± SEM, *n*=10. Means without a common letter differ, $P < 0.05$. * Different from the MZA group, $P < 0.05$.

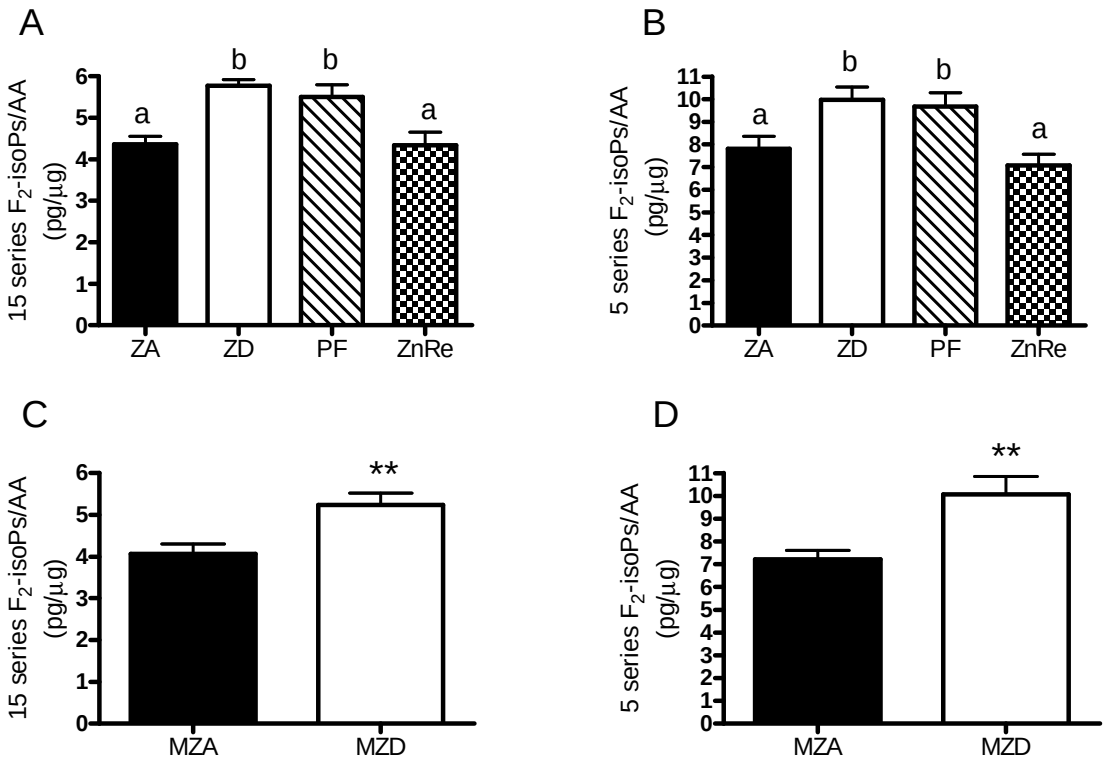

Figure 2.2 Effects of dietary zinc status on (A, C) plasma 15 series F_2-isoprostanes and (B, C) 5 series F_2-isoprostanes in rats. Plasma F_2-isoprostanes were measured as described in *Materials and Methods*. In (A, B), Rats were fed a ZD, PF or ZA diet for 21 d, and then some ZD rats were switched to the ZA diet for 10 d as zinc repletion. In (C, D), rats were fed a MZD or MZA diet for 42 d. Values are means ± SEM, *n*=10. Means without a common letter differ, $P < 0.05$. * Different from the MZA group, $P < 0.05$.

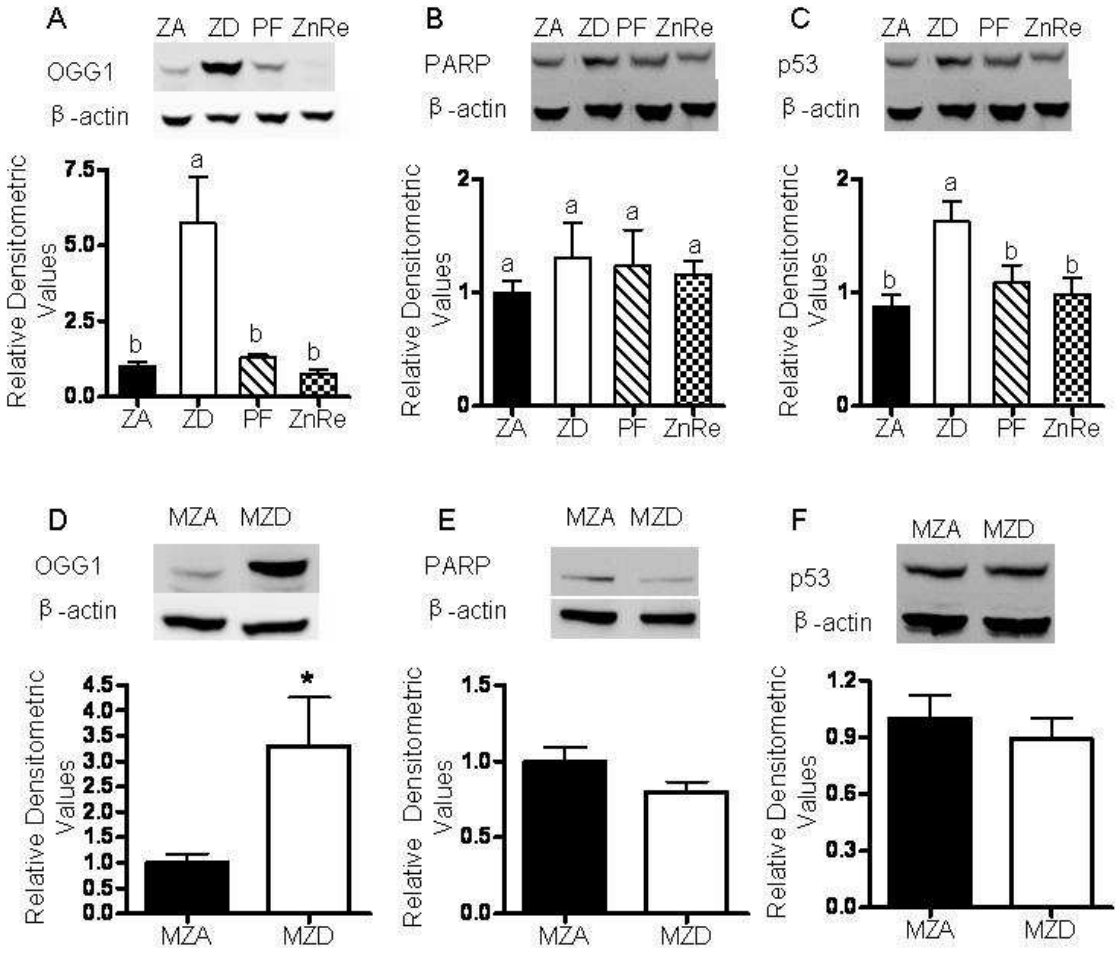

Figure 2.3 Effects of dietary zinc status on the protein levels of DNA repair proteins (A, D) OGG1, (B,E) PARP and (C,F) p53 in rat livers. Protein levels of DNA repair proteins were measured in liver lysates as described in *Materials and Methods.* Relative abundance of each protein was determined by densitometry analysis. Representative Western blots are shown in the inserts. Values are means ± SEM, *n*=6 independent animals. In (A, B, C) Rats were fed a ZD, PF or ZA diet for 21 d, and then some ZD rats were switched to the ZA diet for 10 d as zinc repletion. Means without a common letter differ, $P<0.05$. In (D, E, F), rats were fed a MZD or MZA diet for 42 d. * Different from the MZA group, $P < 0.05$.

Chapter 3 Marginal zinc deficiency increases the susceptibility to oxidative DNA damage and alters DNA repair protein expression in the prostates of voluntary wheel running rats

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3.1 Abstract

Approximately 12% of Americans do not consume Estimated Average Requirement for zinc and could be at risk for marginal zinc deficiency. Zinc functions in antioxidant defense and DNA repair, and could be important for prostate health. We hypothesized that marginal zinc deficiency sensitizes the prostate to oxidative stress and DNA damage. Rats were fed a zinc-adequate (ZA, 30mg Zn/kg) or marginally zinc-deficient (MZD, 5-6mg Zn/kg) diet for 6wk. MZD increased p53 and PARP expressions but not DNA damage in the prostates, as indicated by 8-hydroxy-2-deoxyguanosine (8-OHdG). To examine the susceptibility to exogenous oxidative stress, rats fed a ZA or MZD diet were assigned to exercise (EXE) or sedentary (SED) group for 9wk. MZD or EXE alone did not affect DNA damage in the prostates; however, MZD+EXE increased DNA damage in the dorsolateral lobes. PARP and p53 expressions were not further induced with DNA damage with MZD+EXE, suggesting that MZD interferes with DNA repair responses to stress. Finally, addition of phytase to MZD diet successfully restored zinc status and reduced DNA integrity. Overall, the current study suggests that marginal zinc deficiency sensitizes the prostate to oxidative stress, and demonstrates the importance of maintaining optimal zinc nutriture in physically active populations.

Key words: Zinc deficiency, Prostate, DNA damage, 8-Hydroxy-2'-deoxyguanosine, Exercise, DNA repair

3.2 Introduction

Approximately 12% of Americans do not consume the Estimated Average Requirement (EAR) for zinc , thus a significant proportion of the U.S population could be at risk for marginal zinc deficiency (147). The prostate contains the highest concentration of zinc (Zn) of any soft tissue and secretes high amounts of zinc in prostatic fluid (92). Zinc concentrations in malignant prostate tissues are about 10-25% of that found in healthy prostates (93), suggesting that high zinc concentrations may be essential for the maintenance of prostate health. However, the specific functions of zinc in the prostate and the mechanisms by which zinc maintains prostate health are still unclear.

Zinc is an important element in numerous transcription factors, antioxidant defense enzymes and DNA repair proteins such as p53. In particular, zinc may function in cells to help maintain DNA integrity. Low cellular zinc increases oxidative stress and impairs DNA binding activity of p53 (57, 73). Thus, several different mechanisms may be involved in processes leading to impaired DNA integrity with zinc deficiency *in vivo*: 1) zinc deficiency may increase oxidative stress which may directly cause DNA damage; 2), zinc deficiency may impair DNA damage repair responses (142).

One functional consequence of zinc deficiency is an increased susceptibility to exogenous oxidative stresses such as hyperoxia and endotoxin challenge (9, 10). We have previously shown that zinc deficiency increases oxidative stress and DNA damage in prostate epithelial cells *in vitro* (70). We have also reported that severe zinc depletion (<1ppm Zn in diet) *in vivo* increases oxidative stress and DNA damage (152). Although marginal zinc deficiency is more physiologically relevant to human zinc deficiency than severe zinc deficiency (153), many of the studies only used severe zinc depletion models. Very few *in vivo* studies have been done to examine the effects of marginal zinc deficiency on animal's susceptibility to oxidative stress. The goal of the current study was to examine the effects of marginal zinc depletion on DNA integrity in the prostate both alone and in the presence of chronic exercise as an exogenous oxidative stress. We hypothesized physically active animals under zinc

deficient conditions may be more susceptible to oxidative stress and have impaired DNA repair, which would cause persistent DNA damage in the prostates.

In addition, since the prostate lobes differ markedly in zinc concentration, function and embryological origin (109, 110), we analyzed the dorsolateral and ventral lobes to examine lobe-specific effects. Finally, we added phytase to the marginally zinc-deficient diet to examine whether phytase supplementation to a low-zinc diet could reverse the deleterious effects of marginal zinc depletion on DNA integrity. Since the bioavailability of zinc in animal sources of foods is much higher than the plant sources of food, populations with negligible intake of animal proteins, such as vegetarians may be at risk of marginal zinc deficiency. Phytate, which is present in high amounts in plant sources of food, is one of the major inhibitors for zinc absorption. Therefore, providing phytase supplementation to those populations susceptible to marginal zinc deficiency may increase zinc absorption and improve zinc status. The current study is one of the first to examine the *in vivo* effects of dietary marginal zinc depletion and exercise on DNA integrity in the prostate and highlights the importance of maintaining adequate-zinc status in physically active populations.

3.3 Material and Methods

Animals

Marginal zinc depletion study. Male Sprague-Dawley rats (12/group, 5 wk old, ~ 110g) from Charles River Laboratories (Wilmington, MA) were acclimated for 1 wk to the temperature- and humidity-controlled environment with a 12-h-dark: light cycle. They were maintained in polycarbonate cages. Rats were randomly assigned to one of two dietary treatments: zinc-adequate diet (ZA, 30 mg Zn/kg) or marginally zinc-deficient diet (MZD, 5-6 mg Zn/kg).

Zinc & chronic exercise interaction study. Male Sprague-Dawley rats (12/group, 4 wk old, 125-150 g) from Charles River Laboratories, Inc. were housed individually in polycarbonate cages and acclimated for 2 wk to the temperature- and humidity-controlled environment with a 12-h-dark: light cycle. Rats were then divided into voluntary wheel running (EXE) or sedentary (SED) groups based on equal average body weight. Rats in EXE group were assigned to cages equipped with 345 mm

(diameter) running wheels (Mini Mitter Co., Bend, OR), and were allowed unrestricted access to the running wheels. Each cage was fitted with a magnetic switch to allow for the counting of wheel revolutions using Vital View 3000 software (Mini Mitter Co.). Animal were fed a zinc-adequate (ZA, 30mg Zn/kg) or marginal zinc-deficient (MZD, 5-6mg Zn/kg) diet. To further examine the effects of phytase on zinc status and DNA integrity in the prostate, 1,500 phytase units/kg diet (Natuphos, BASF Corporation, Florham Park, NJ) was added to the MZD diet and fed to the rats in both EXE and SED groups (MZD+P).

For all studies, diets were based on modified AIN-93M diets (154) formulated with egg white rather than casein, and zinc was provided as zinc carbonate (Research Diets Inc, New Brunswick, NJ). Deionized water was provided as drinking water. Rats were killed following anesthesia with isoflurane overdose (1-5%; Henry Schein; Melville, NY). Diet intakes and body weights were measured twice per week. The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at U.S. Army Research Institute of Environmental Medicine (USARIEM) and Oregon State University, and animals were maintained in accordance with IACUC's guidelines for the care and use of laboratory animals.

Tissue and blood collection

Blood samples were collected by cardiac puncture into trace element-free vials containing EDTA. Plasma was separated immediately and frozen at -80ºC until analysis. Samples of prostate (whole prostate or microdissected dorsolateral and ventral prostate lobes) were dissected and half were immediately snap frozen at -80℃ and the other half were stored in RNAlater (Am bion, Austin, TX) until analysis.

Zinc analysis

Plasma, ventral, dorsolateral lobe and whole prostate (n=12) zinc concentrations were determined by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES; Teledyne Leeman Labs, Hudson, NH) with a minor modification of a previously described method (155). Plasma or prostates were digested in 1 ml 69%-70% nitric acid overnight. Following digestion, samples were diluted 10 times with

water treated with chelex 100 resin (Bio-Rad Laboratories, Hercules, CA) and analyzed by ICP-OES against known standards (152).

DNA damage analysis

Comet assay in peripheral blood cells: Single-strand breaks in peripheral blood cells (n=10) were determined by alkali single-cell gel electrophoresis (Comet assay) as described by Singh et al. (156). Blood cell pellets isolated from fresh blood were mixed with Low Melting Point agarose and applied onto microscope slides (Trevigen, Gaithersburg, MD). DNA in lysed cells was allowed to unwind in alkali buffer (0.3 M NaOH, 1mM EDTA) for 20 min. Samples then underwent electrophoresis for another 20 min at 300 mA, 25 V. Immediately before scoring, nuclear material was stained with 20 µL/circle ethidium bromide (20 mg/L). Comet measurements were made by image analysis using Nikon E400 fluorescent microscope and Comet Assay III software (Perceptive Instruments, UK). Images of 100 randomly selected nuclei (50 nuclei from each of two replicate slides) were analyzed from each sample. The comet measurements of tail moment were recorded and used to indicate DNA damage in rat peripheral blood cells. One comet slide was made for each rat, and on each slide 50 comets were scored blindly for tail moments. Results are presented as fold of the tail moments to the zinc-adequate group.

ELISA analysis of 8-hydroxy-2-deoxyguanosine in the prostate: DNA was isolated using a chaotropic sodium iodide method as described by Helbock et al (170). 8-OHdG levels (n=4) were measured in DNA extracted from rat prostate using 8-OHdG ELISA kit (Japan Institute for the Control of Aging, Tokyo, Japan) following manufacturers instructions. Briefly, 50 µl of isolated DNA sample or standard was added to each well, and the plate was read at 450 nm by Spectra Max plate reader (Molecular Device, Sunnyvale, CA).

Quantitative Real-Time PCR analysis

The mRNA abundance of p53 and PARP in the prostate was measured by quantitative Real-Time PCR (qRT-PCR). Total RNA was extracted from prostate tissue using an RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). First-strand cDNA was reverse transcribed from the isolated RNA

using Superscript First-Strand Synthesis System (Invitrogen Corporation, Carlsbad, CA). The primers for the measured transcripts were: p53 forward 5'-GCGTTGCTCTGATGGTGA-3'; p53 reverse 5'-CAGCGTGATGATGGTAAGGA-3'; PARP forward 5'-TGTGAACTCCTCTGCACCAG-3'; PARP reverse 5'-AGCTGAGGC AGACACATCC-3'; 18s forward 5'-GGACCAGAGCGAAAGCATTTGC-3'; 18s reverse 5'- CGCCAGTCGGCATCGTTTATG-3'. The annealing temperatures for all transcripts were 58ºC, and the cycle number was 40. The PCR reactions were performed using DyNAmo HS SYBR Green qPCR kit (New England BioLabs, Ipswich, MA) as described by the manufacturer. A standard curve was generated from serial dilutions of purified plasmid DNA that encoded for the gene of interest. Data represent averaged copy number normalized to the 18s housekeeping gene.

Western analysis of DNA repair proteins

Nuclear and cytosolic protein was extracted from frozen prostate using Nuclear Extract Kit (Active Motif, Carlsbad, CA) as described by the manufacturer. Protein concentrations were determined using DC Protein Assay (Bio-Rad Laboratories). Proteins (30µg/lane) were separated by SDS-PAGE on a 4-12% bis-Tris gel (Invitrogen Corporation) and transferred to nitrocellulose membrane (Bio-Rad Laboratories). Equal protein loading were confirmed with β-actin levels. After blocking the membranes, the primary antibodies used for detection were mouse anti-p53 (Ab-1, Calbiochem, San Diego, CA), mouse anti-Poly-ADP ribose polymerase (PARP, BD Pharmingen, San Jose, CA), and mouse anti-β-actin (Sigma-Aldrich, St.Louis, MO). Bound antibodies were detected using either goat anti-mouse IgG-HRP or goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA), and developed with SuperSignal West Femto chemiluminescent substrate (Pierce, Rockford, IL). Images were acquired on Alpha Innotech photodocumentation system (Alpha Innotech, Hayward, CA) and analyzed using Image J 1.37v software (NIH, Bethesda, MD).

Statistical analysis

Statistical analysis was performed with the use of PRISM (version 4.0; GraphPad Software, San Diego, CA). Student's t-test was used for two-group comparisons. Main effects of exercise, diet and interaction factors were analyzed by two-way

ANOVA. Pair-wise comparisons between groups with the same exercise level or the same diet were analyzed by Bonferroni's post-hoc test. Appropriate data transformation was taken to account for unequal variances Differences were considered statistically significant at $P<0.05$. Values were means ± SEM (standard error of the mean) unless otherwise indicated.

3.4 Results

Body weights

Marginal zinc depletion did not change the food intake in all studies (data not shown). By the end of the study, average body weights in the EXE group (322.3 ± 5.4 g) were significantly lower than in the SED group (360.7 ± 5.2 g, $P<0.05$), and the body weight in the MZD group (312.8 ± 5.3 g) was significantly lower than the ZA group (368.7 ± 6.8 g, $P<0.001$).

Effects of marginal zinc depletion on DNA damage and DNA repair

Marginal zinc depletion alone for 6 wk significantly reduced plasma zinc concentrations by 22% (MZD vs ZA, 2.54 ± 0.22 vs 3.19 ± 0.22 μg/mL; $P<0.05$) and prostate zinc concentrations by 30% compared to the ZA rats (**Figure 3.1A**, $P<0.05$).

To examine the effects of marginal zinc depletion on DNA integrity, two types of DNA damage were measured: DNA strand-breaks in peripheral blood cells by Comet assay and oxidative DNA damage in prostates by 8-OHdG ELISA. The tail moments of peripheral blood cells in the MZD group were 20% higher than in the ZA group (**Figure 3.1C**, $P<0.05$), and the prostate 8-OHdG concentrations in the MZD group were 32% higher (*NS*) than in the ZA group (**Figure 3.1B**, $P=0.20$). However, the 8-OHdG concentrations in the prostate were strongly and significantly correlated to the tail moments of peripheral blood cells (**Figure 3.1D**, r=0.66 $P=0.03$).

Since DNA repair functions are crucial for maintaining DNA integrity, we examined the effects of marginal zinc depletion on DNA repair proteins PARP and p53 in the prostates. PARP is a zinc-containing protein and plays an essential role in DNA repair (171). The abundance of PARP and p53 mRNA in the prostates of the MZD rats were significantly higher than the ZA rats (**Figure 3.2A&B**, $P<0.05$).

Consistent with the mRNA expressions, both p53 and PARP protein levels in the prostates of the MZD rats were significantly higher than the ZA rats (**Figure 3.2D&E**, *P*<0.05). These data indicate that marginal zinc depletion activates DNA repair activities in the prostate, which indirectly suggests DNA damage stress is present in the prostate with marginal zinc deficiency. Activation of these repair systems might also partially account for why we did not observe significant increases of oxidative DNA damage in the prostates of the MZD rats.

Effects of marginal zinc depletion on chronic exercising rats

We performed another marginal zinc depletion study in voluntary wheel running rats, and examined the combined effects of marginal zinc depletion and chronic exercise on DNA integrity in the prostate. Zinc concentrations in plasma and prostate dorsolateral lobes of the MZD rats were significantly lower than the ZA rats (**Table 3.1,** *P*<0.05). In contrast, zinc concentrations in prostate ventral lobes were not affected by marginal zinc depletion, indicating that prostate dorsolateral lobes, which retain higher zinc concentrations than the ventral lobes, were more susceptible to dietary marginal zinc depletion. Exercise alone had no effects on tissue zinc concentrations (Table 1 Exercise, *P*>0.05).

We then evaluated oxidative DNA damage in the dorsolateral and ventral lobes, and found that 8-OHdG concentrations in prostate dorsolateral lobes of the MZD rats were higher than the ZA rats in the EXE group (206% of the ZA rats, **Figure 3.3A**, *P*<0.05) but not in the SED group. Neither marginal zinc deficiency nor chronic exercise alone had any effects on prostate oxidative DNA damage (*P*>0.05). In addition, 8-OHdG concentrations in prostate ventral lobes were not affected by marginal zinc depletion and/or chronic exercise (**Figure 3.3B**, *P*>0.05). Collectively, these results indicate that marginal zinc depletion increases rats' susceptibility to chronic exercise-induced oxidative stress and impairs DNA integrity specifically in prostate dorsolateral lobes. Since zinc concentrations and 8-OHdG concentrations were only altered in prostate dorsolateral lobes, we examined DNA repair protein specifically in the dorsolateral lobes. Within the SED group, p53 and PARP mRNA abundance in the MZD rats were significantly higher than in the ZA rats (**Figure 3.4A&B**, *P*<0.05), which is consistent with what we found in the whole prostates of

the marginal zinc depletion study (Figure 2A&B). Although chronic exercise alone did not significantly increase p53 expression (ZA of EXE vs ZA of SED, P=0.17), it did significantly increased PARP expression (P<0.05). Interestingly, despite increased DNA damage, marginal zinc depletion in the EXE group failed to further increase p53 and PARP gene expressions (within the EXE group, MZD vs ZA, P>0.05). Thus the ability of the prostate cells to respond to additional stress and DNA damage may have been compromised. Collectively, these results suggest that marginal zinc depletion may compromise the protective responses of the prostate to chronic exercise-induced oxidative stress and interfere with the activation of DNA repair protein expressions, and contribute to the effects of marginal zinc depletion and chronic exercise on prostate DNA integrity.

Effects of phytase supplementation on prostate DNA integrity

We found that addition of phytase to the MZD diet increased zinc concentrations in plasma (in press, J Nutr Biochem 2009) prostate dorsolateral lobes to the levels in the ZA rats (**Figure 3.5A**, P<0.05). Along with elevated zinc concentrations in prostate dorsolateral lobes, oxidative DNA damage was significantly reduced to the ZA levels in the MZD+P rats (**Figure 3.5B,** P<0.05). These results indicate that phytase supplementation effectively reverses the deleterious effects of marginal zinc depletion on DNA integrity in the prostate dorsolateral lobes.

3.5 Discussion

The present study shows that neither marginal zinc deficiency nor chronic exercise caused oxidative damage in the prostates; however, the combined effects of low zinc with chronic exercise significantly increased oxidative DNA damage in the dorsolateral lobes of the prostate. Importantly, the dorsolateral lobe is the region that accumulates the highest levels of zinc in the prostate and is also the region most susceptible to prostate cancer (108, 109). The interactive effect of marginal zinc depletion and chronic exercise could be due to increases in oxidative stress in combination with the impairment of DNA damage responses through interference with the activation of p53 and PARP. In addition, we also show that phytase

supplementation effectively maintained zinc levels in the prostate and prevented the impairment of DNA integrity in the prostate of rats fed marginal zinc deficient diets. Overall, these results suggest that zinc deficiency may markedly affect one's response to exogenous oxidative stresses and DNA damaging agents and potentially highlights the importance of maintaining optimal zinc nutriture in physically active populations to prevent oxidative DNA damage in the prostate.

Prostates contain the highest zinc concentration of any soft tissue (92). It has been proposed that maintaining high zinc content is essential for prostate health, and losing the ability to accumulate high zinc levels may contribute to the prostate malignancy (94-98). However, prostate lobes have substantially different zinc concentrations and also have different susceptibility to malignancy. In human prostates, the peripheral zone contains much higher zinc than the other zones, and 80% prostate malignancies develop in this zone (108). The dorsolateral lobe of rat prostate is embryologically homologous to the peripheral zone of the human prostate (109) and retains higher levels of zinc than the ventral lobe (110). Therefore, dorsolateral and ventral lobes may have differential sensitivity to marginal zinc depletion. The current study confirms that there is a differential response to dietary zinc depletion between the dorsolateral and ventral lobes of the prostates. The ventral lobe is able to maintain zinc concentrations during marginal zinc depletion, whereas the dorsolateral lobe only preserves 30-40% of its normal zinc concentration. Moreover, marginal zinc depletion and chronic exercise together only significantly increase oxidative DNA damage in dorsolateral lobes but not ventral lobes. Altogether, the prostate dorsolateral lobes, not ventral lobes, are susceptible to marginal zinc depletion, oxidative stress and oxidative DNA damage. One factor that may determine the susceptibility to zinc deficiency is the prostate's ability to maintain zinc homeostasis. It has been proposed that the dysregulation of zinc transporters in the prostate may impair zinc homeostasis and contribute to prostate malignancies (93, 95, 98). Further examination of the mechanisms regulating zinc concentrations and compartmentalizing in the prostate during zinc deficiency and prostate cancer progression is an important area for future studies.

Previous studies have shown that dietary zinc depletion increases DNA strand breaks in rat peripheral blood cells through increases in oxidative stress and

interference of DNA repair proteins including p53 and PARP (152). *In vitro* we have found that p53 binding activity is impaired in prostate epithelial cells with zinc depletion (58, 70). However, the current study found that in the prostate dietary marginal zinc depletion alone did not significantly increase oxidative DNA damage, despite a 30% reduction in prostate zinc concentrations. Similarly, chronic exercise alone did not increase oxidative DNA damage in the prostates. These are not entirely surprising because of the possible health-beneficial effects of chronic exercise. Indeed, vigorous exercise increases oxidative damage and the generation of ROS/RNS (77-80) by enhancing oxygen consumption and inducing inflammatory responses as a result of tissue injury (172). However, it has been shown that the long-term chronic exercise training may beneficially reduce mitochondrial hydrogen peroxide (88, 89) in the muscle tissue. Therefore, chronic exercise alone may not impair DNA integrity.

Although neither marginal zinc deficiency nor chronic exercise alone increased oxidative DNA damage, a combination of both factors increased oxidative DNA damage in the prostate dorsolateral lobes. One possible mechanism is that the collective oxidative stress caused by both marginal zinc depletion and chronic exercise exceeds the capacity of antioxidant defense to remove the free radicals and DNA repair functions to repair DNA damage, resulting in oxidative DNA damage. It has been established that zinc has antioxidant capacity, and that zinc depletion in rats generates carbon-centered free radicals in lung microsomes (4) and oxidatively modified proteins and lipids in various tissues (5-8). Studies in our lab have shown that both severe (152) and marginal zinc depletion (unpublished data) increases F_2-isoprostanes in rat plasma. The mechanisms for the antioxidant functions of zinc include: 1) zinc protects the sulfhydryl groups from oxidation, thereby maintains normal functions of proteins such as 5-aminolaevulinate dehydratase (11); 2) zinc maintains the reductive intracellular environment though the modulation of thiol status (7, 29); 3) zinc antagonizes the activity of bivalent transition metals including iron and copper, and prevents the deleterious free-radical reactions (e.g. Fenton reaction); 4) zinc regulates the expression of metallothionein (20, 22) and CuZn SOD activity.

A second mechanism for the interactive effects of marginal zinc depletion and chronic exercise on DNA integrity could be related to the DNA repair functions which

are insufficiently activated and unable to respond to DNA damage. The current study specifically examined the DNA repair proteins p53 and PARP in the prostates. p53 is a zinc-containing protein, and functions in DNA repair, cell proliferation and cell death (65). Zinc is located in the DNA binding domain of p53 and is essential for the DNA binding activity of p53. Although p53 expression is induced in zinc depleted cells and animals (57, 65), the DNA binding activity is impaired by zinc deficiency (58, 70). PARP is also a zinc-containing protein, and PARP-like zinc finger (zf-PARP) is essential for the recognition of DNA strand breaks and DNA binding of PARP (167). PARP binds to DNA single-strand breaks (SSB) which are created during Base Excision Repair (BER) through its zinc finger motif and recruits other DNA repair factors (e.g. XRCC and DNA ligase) to the nick to finish BER (166, 167). One human study revealed a positive correlation between cellular poly(ADP-ribosyl)ation capacity and zinc status in PBMC, indicating that zinc could be required for PARP activity (168). PARP is activated by the accumulation of DNA strand breaks and 8-OHdG in nuclear DNA and play pivotal roles in DNA-repair and cell check-point pathways (171, 173). If cells possess normal DNA damage responses, increases in oxidative stress and DNA lesion will effectively activate p53 and PARP expressions to repair or eliminate DNA damage. With marginal zinc depletion, we measured increased expressions in p53 and PARP. However, we did not detect significant increases in p53 and PARP in the marginal zinc-depleted EXE rats in comparison with the zinc-adequate EXE rats, although oxidative DNA damage was substantial increased by marginal zinc depletion in the EXE rats. These data indicate that marginal zinc depletion may have impaired DNA damage responses in the prostates of chronic exercising rats and increased the susceptibility of the prostate to oxidative DNA damage.

High phytate consumption markedly decreases the bioavailability of dietary zinc by forming an insoluble phytate-zinc complex with zinc that is difficult to absorb in the gastrointestinal tract. Phytate is a major factor that accounts for why vegetarians or people in developing countries are at high risk of zinc deficiency. It has been demonstrated that dietary microbial phytase supplementation in animals fed zinc deficient diets improves zinc status (174-177). The current study further shows that phytase supplementation improved zinc status and reversed the effects of marginal

zinc depletion on DNA integrity in the prostates, and suggests that phytase supplementation might be an effective intervention strategy to prevent zinc-deficiency induced DNA damage in populations habitually consuming high phytate, low-zinc diets.

Overall, the current study shows an important interactive effect of marginal zinc deficiency and chronic exercise on impairing DNA integrity, suggesting that marginal zinc depletion sensitizes the prostates to oxidative stress and increases oxidative DNA damage. The current study also points out the importance to maintain optimal zinc nutriture in physically active populations and highlights the importance of zinc in prostate health.

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List of Abbreviations

8-OHdG, 8-hydroxy-2'-deoxy-guanosine; BER, base excision repair; EAR, Estimated Average Requirement; EXE, voluntary wheel running group; ZA, zinc-adequate group; MZD, marginally zinc-deficient group; MZD+P, marginally zinc-deficient group with phytase supplementation; PARP, poly ADP ribose polymerase; SED, sedentary.

Table 3.1 Zinc concentrations in plasma, prostate ventral and dorsolateral lobes of the EXE or SED rats fed the MZD or ZA diet[1]

	EXE		**SED**		***P*-value**		
	MZD	ZA	MZD	ZA	Inter-action	Exer-cise	Zn
Plasma (μg/ml)	0.52±0.06***	1.18±0.06	0.60±0.04***	1.06±0.05	0.06	0.61	<0.0001
Prostate ventral lobe (mg/kg)	12.90±1.23	12.90±0.42	10.93±0.93	12.00±1.53	0.62	0.19	0.62
Prostate dorsolateral lobe (mg/kg)	58.34±1.28**	237.13±1.10	57.54±1.35*	155.59±1.04	0.40	0.34	0.0015

[1] Values are means mean ± SEM (n=6). Main effects of exercise, zinc and interaction were determined by two-way ANOVA with Bonferroni post-hoc test.
* P<0.05, **, P<0.01, ***, P<0.001,means differ from the ZA rats within the same EXE or SED group.

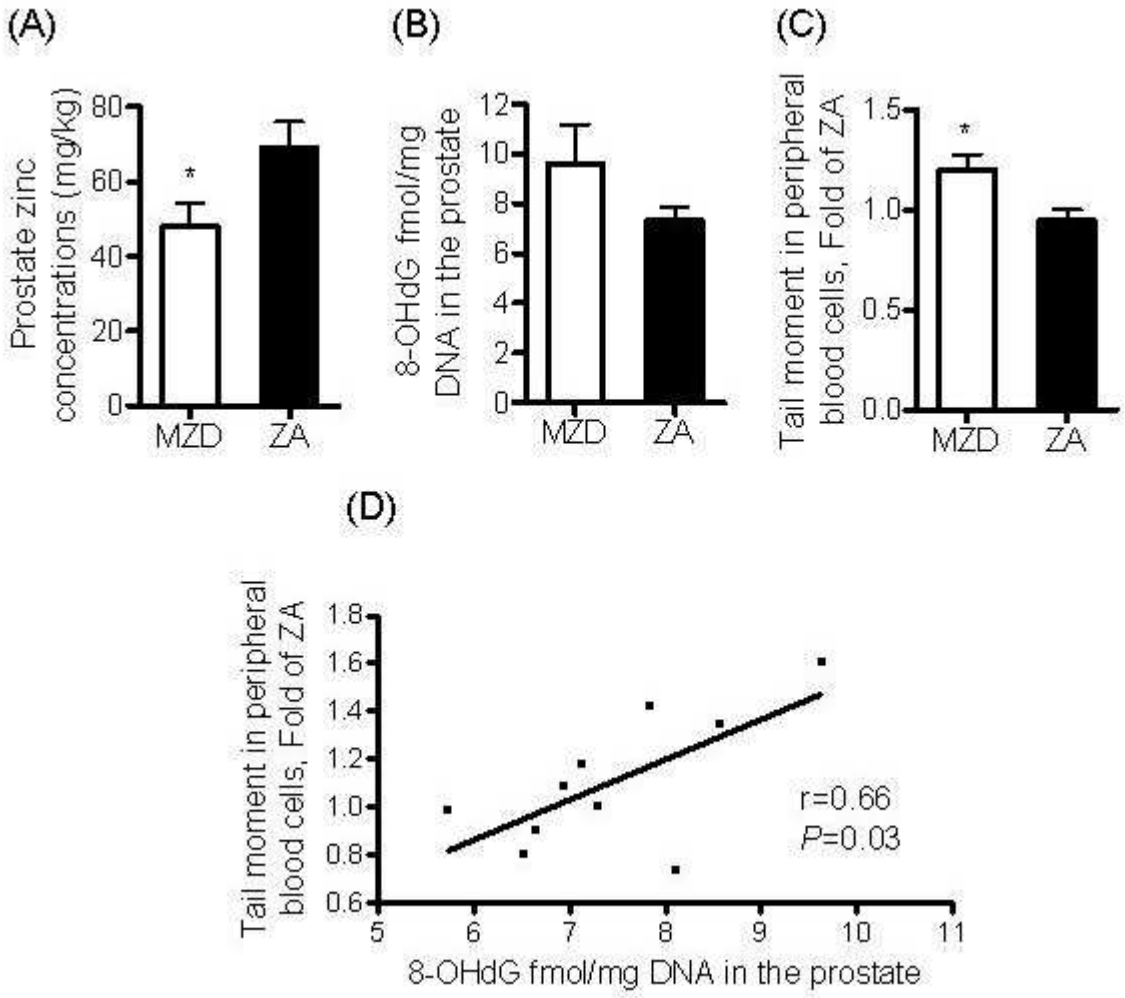

Figure 3.1 Effects of marginal zinc depletion on (A) zinc concentrations and (B) oxidative DNA damage in the whole prostates, and (C) DNA strand breaks in peripheral blood cells of rats. Rats were fed a MZD or ZA diet for 6 weeks. Oxidative DNA damage was measured by 8-OHdG ELISA and DNA strand breaks were measured by Comet assay. (D) Prostate 8-OHdG concentrations were plotted as a function of tail moment of peripheral blood cells, with correlation "r" and P-value. Values are means ± SEM (n=10). *, $P<0.05$, means differ from the ZA group.

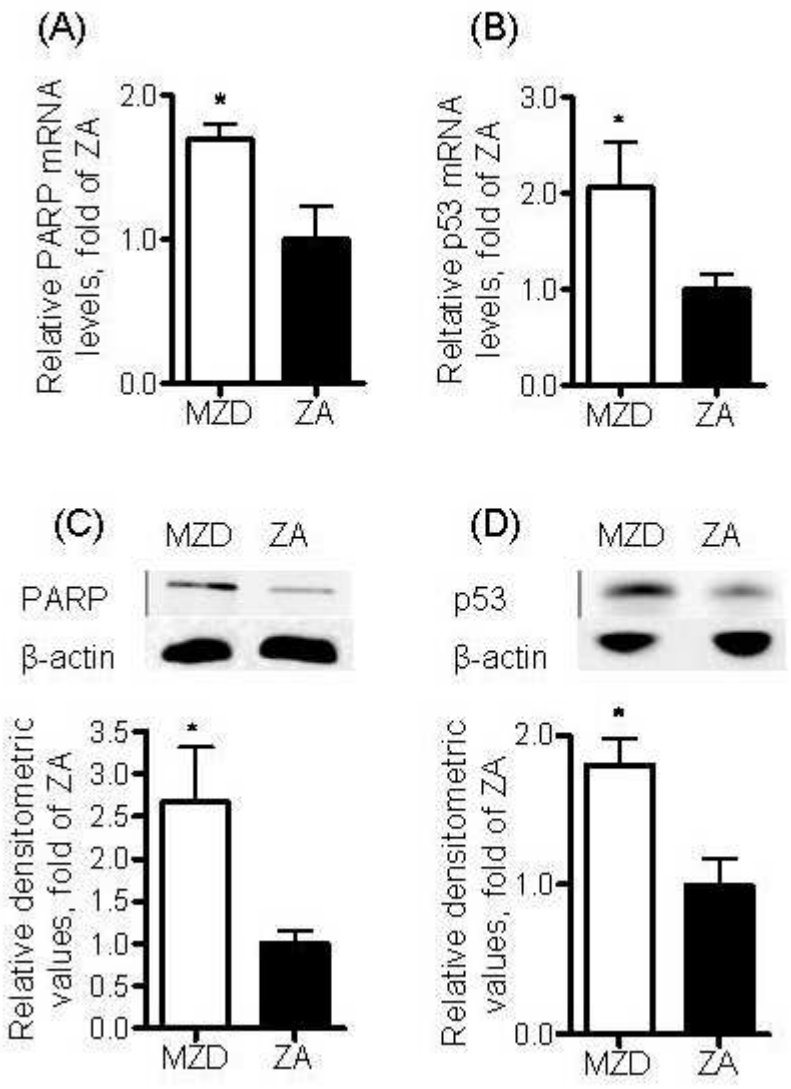

Figure 3.2 Effects of marginal zinc depletion on (A, B) gene expressions and (C, D) proteins levels of DNA repair proteins, p53 and PARP, in the whole prostate. Rats were fed a MZD or ZA diet for 6 weeks. (A, B) Results of qRT-PCR are presented as relative transcripts copy number normalized to 18s transcripts. (C, D) Relative abundance of each protein was determined by densitometry analysis with normalization to β-actin. Representative Western blots are shown in the inserts. Values are means ± SEM (n=6). *, $P < 0.05$, means differ from the ZA group.

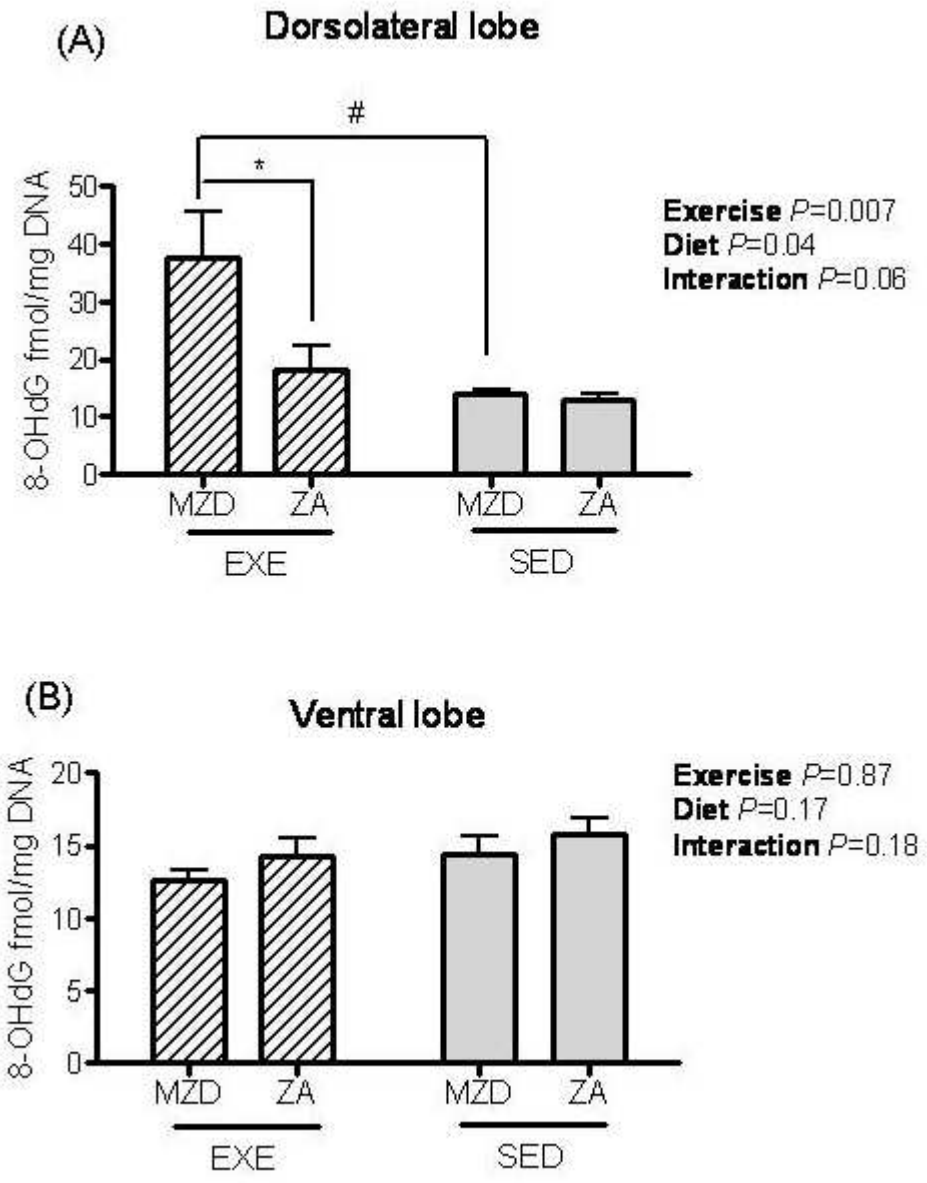

Figure 3.3 Effects of marginal zinc depletion and chronic exercise on 8-OHdG levels in rat prostate (A) dorsolateral and (B) ventral lobes. Voluntary wheel running or sedentary rats were fed a MZD or ZA diet for 9 weeks. Oxidative DNA damage was measured by 8-OHdG ELISA. Values are means ± SEM (n=4). *, $P < 0.05$, means differ between groups with the same exercise levels. #, P<0.05, means differ between groups with the same diet. P-values for the main effects of exercise, zinc and interaction are listed in the figure.

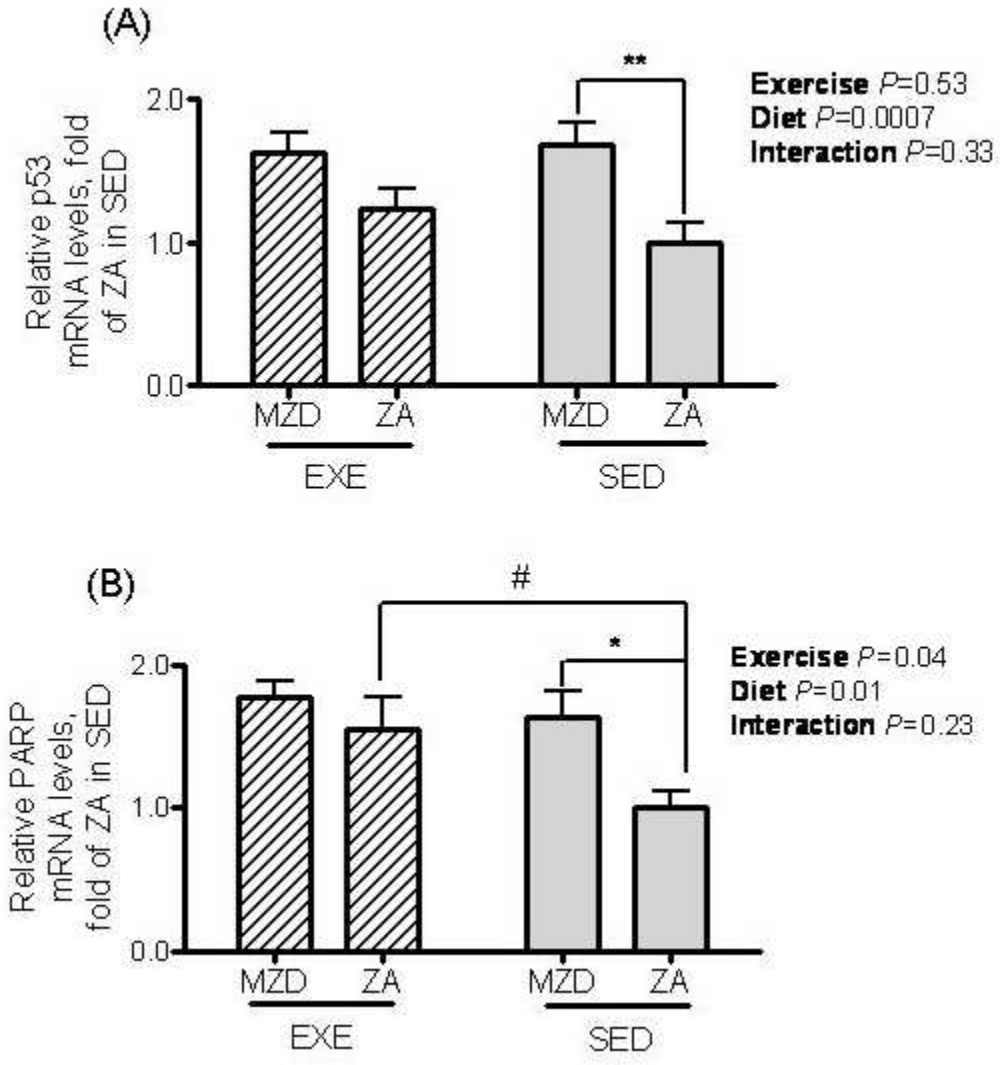

Figure 3.4 Effects of marginal zinc depletion and chronic exercise on DNA repair genes (A) p53 and (B) PARP expression in the dorsolateral lobes of rats. Voluntary wheel running or sedentary rats were fed a MZD or ZA diet for 9 weeks. Values are means ± SEM (n=6). *, $P < 0.05$, ** P<0.01, means differ between groups with the same exercise levels. #, P<0.05, means differ between groups with the same diet. P-values for the main effects of exercise, zinc and interaction are listed in the figure.

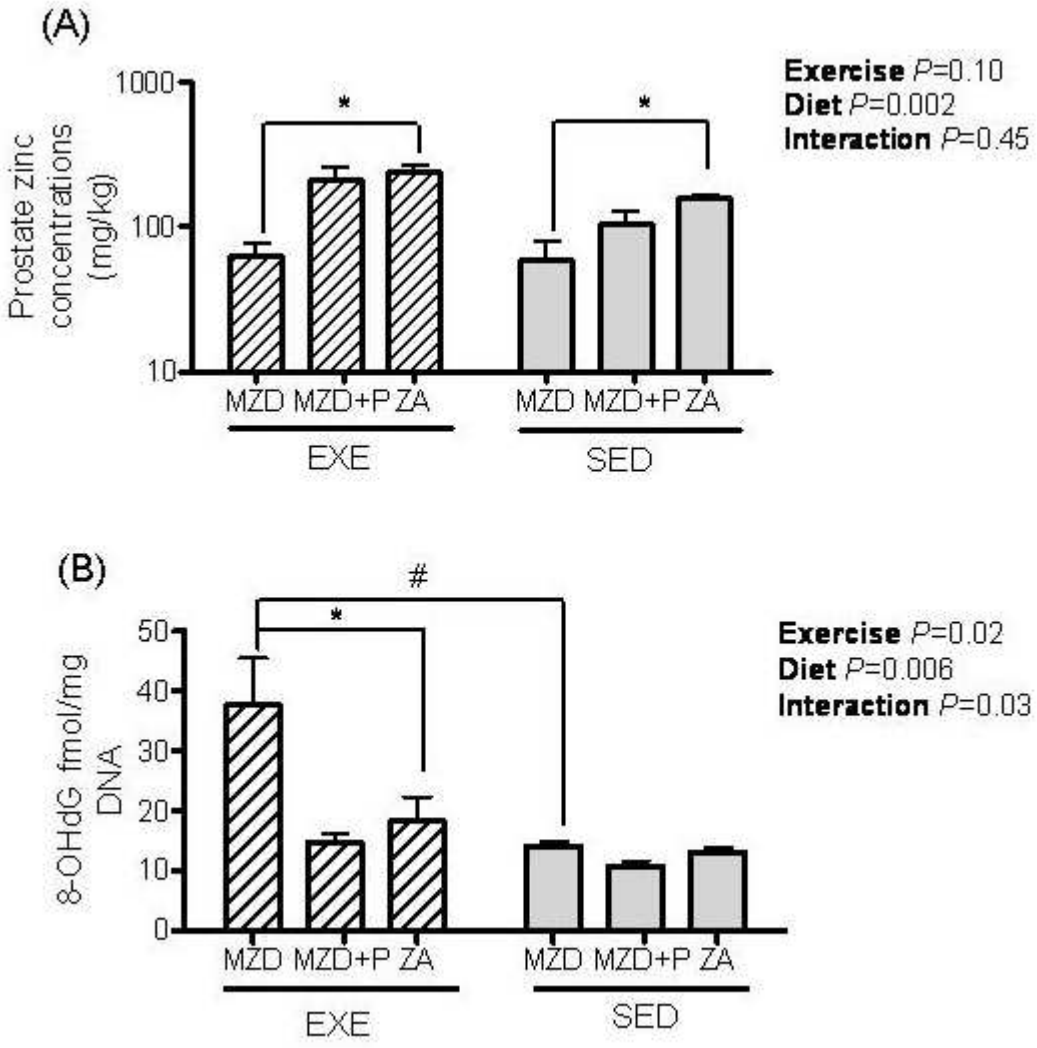

Figure 3.5 Effects of phytase supplementation on (A) zinc concentrations and (B) 8-OHdG levels in the prostate dorsolateral lobes. Voluntary wheel running or sedentary rats were fed a MZD, MZD+P or ZA diet for 9 weeks. Oxidative DNA damage was measured by 8-OHdG ELISA. Values are means ± SEM (n=4). *, $P < 0.05$, means differ from the ZA group within the same exercise levels. #, $P<0.05$, means differ between groups with the same diet. P-values for the main effects of exercise, diet and interaction are listed in the figures.

Chapter 4 Dietary zinc restriction and repletion affects DNA integrity in healthy adult male volunteers

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4.1 Abstract

Background: Zinc plays an important role in antioxidant defense and the maintenance of cellular DNA integrity. However, no experimental human studies have been performed to examine the role of zinc status on DNA damage.

Objective: We evaluated the effects of dietary zinc depletion and repletion on DNA strand breaks, oxidative stress and antioxidant defenses in healthy male volunteers.

Design: Nine healthy men with reported mean daily zinc intakes >11 mg/d were recruited. Subjects completed three consecutive dietary periods: baseline (d1 to d13, 11 mg Zn/d), zinc-depletion (d14 to d55, 0.6 mg Zn/d for 1 w and 4 mg Zn/d for 5 w), and zinc-repletion (d56 to d83, 11 mg Zn/d for 4 w with 20 mg supplemental Zn for first 7 d). Blood samples were collected on days 1, 13, 35, 55, and 83. DNA damage in peripheral blood cells, plasma oxidative stress and antioxidant defense biomarkers were assessed.

Results: Dietary zinc depletion (6 w) was associated with increased DNA strand breaks in peripheral blood cells (d13 vs d55, $P<0.05$), alterations which were ameliorated by zinc repletion (d55 vs d83, $P<0.05$). Plasma zinc concentrations were negatively correlated with DNA strand breaks (r=-0.60, P=0.006) during the zinc depletion period. Plasma α- and γ-tocopherol concentrations, plasma total antioxidant capacity and erythrocyte SOD activity did not change significantly; and plasma F_2-isoprostanes, were unaffected by dietary period.

Conclusion: Changes in dietary zinc intake affected DNA single-strand breaks. Zinc appears to be a critical factor for maintaining DNA integrity in humans.

Key words: zinc, DNA integrity, oxidative stress, DNA damage, healthy men, zinc deficiency.

4.2 Introduction

Worldwide, zinc deficiency is an important public health problem that affects approximately 2 billion people who do not ingest adequate amounts of zinc (34). Human zinc deficiency was first described in Iran in the early 1960s by Prasad et al. (32, 33). The lack of validated biomarkers for assessing individual zinc status precludes our ability to clearly identify those with impaired zinc status. Indeed, the estimated prevalence of zinc deficiency in Americans, based on NHANES data, suggests that at least 12% of Americans do not consume the Estimated Average Requirement (EAR) for zinc. Thus, a considerable proportion of the US population could be at risk for marginal zinc deficiency (15). The current indicators for zinc deficiency, such as plasma or hair zinc concentrations, have poor sensitivity and specificity and do not change with marginal zinc deficiency (178). Thus, the identification of sensitive biomarkers of zinc status is a critical issue in the field.

Zinc is the most abundant trace intracellular element and is required for a number of biological functions including reproduction, immune function and defense against free radicals (179, 180). Zinc is a component of >1000 transcription factors including DNA-binding proteins with zinc fingers, and is required in >300 zinc-containing metalloenzymes. The consequences of zinc deficiency in adults have been understudied despite the recognition of symptoms of zinc deficiency for decades. Moreover, a considerable body of evidence suggests that zinc deficiency may increase the risk for some chronic diseases, including cancer (49, 50). This link may be attributed to the role of zinc in antioxidant defense and DNA damage repair. Zinc-depleted cells and experimental animals have greater susceptibility to oxidative stress and elevated concentrations of oxidatively modified proteins, lipids, and DNA damage (5-10, 57, 152, 181). Since zinc also plays essential roles in DNA damage repair (142), DNA damage increases with zinc deficiency may be due to perturbations in oxidative stress as well as impaired DNA repair functions. Overall, zinc deficiency may both increase oxidative stress and impair DNA integrity, thereby increasing the risk of cancer. In vitro and animal studies have clearly demonstrated increased DNA damage and oxidative stress with zinc deficiency (7, 8, 57, 60, 61, 73, 152). However, the extent to which zinc depletion regulates oxidative stress and DNA integrity in

controlled human studies has yet to be comprehensively explored. Thus, the objectives of our study were to examine oxidative stress responses and DNA integrity using a marginal zinc depletion and repletion protocol in healthy men. We hypothesized that marginal dietary zinc restriction would impair DNA integrity and increase oxidative stress in humans, and that these deleterious effects would be reversed by zinc repletion. Collectively, the findings from this study may help identify new biomarkers for marginal zinc deficiency in humans and provide the justification for their use in future human trials.

4.3 Subjects and Methods

Subjects

Healthy males aged 19-50 y were recruited (beginning 1/28/2004) from the Davis and San Francisco area, as reported previously (182) . Men with fasting plasma zinc concentrations >74 μg/dL and reported usual dietary zinc intake ≥ 11mg/d, the recommended daily allowance for men, were considered to be zinc adequate and were invited to participate the study. Dietary zinc intakes were assessed before entry into study using the average of five 24-hour dietary recalls. Exclusion criteria included: vegetarian diet, cigarette smoking, and chronic use of alcohol or prescription drugs, use of illicit drugs, regular consumption of zinc supplements, or consumption of any zinc supplements during the 4 weeks before entering into the study.

All subjects gave written, informed consent to participate in the study. The study protocol was reviewed and approved by the Institutional Review Boards at the University of California, Davis, the Children's Hospital and Research Center of Oakland, the Committee on Human Research at the University of California, San Francisco and Oregon State University. The study was conducted at two study sites, the Ragle Human Nutrition Research Center at UC Davis and the General Clinical Research Center (GCRC) at the San Francisco General Hospital (SFGH), University of California, San Francisco. Three subjects resided at the GCRC in San Francisco for the study duration. The other six subjects followed a free-living protocol, whereby they consumed either all weekday meals at the Ragle Human Nutrition Research

Center or weekday lunch meals at the GCRC (breakfast and dinner were provided for home consumption). All weekend meals were provided for consumption at home.

Study Design

To obtain altered zinc status, the subjects completed a period of marginal dietary zinc depletion followed by a period of dietary zinc repletion (**Figure 4.1,** adapted from (182)), using protocols previously used by Ruz et al with slight modification (183). The study was divided into three diet periods. During Diet Period (DP) 1 (Baseline period, d1-13), an adequate zinc diet (11 mg Zn/day) was given for 13 days to ensure adequate zinc status. During Diet Period 2 (Zinc depletion period, d14-55), the subjects were given a liquid diet containing 0.6 mg Zn/day for 7 days followed by a low zinc diet (4 mg Zn/day) for another 35 days. Phytate (1.3 g/day, Sigma, St. Louis, MO) was added to the diet for the first 21 days of Diet Period 2 to inhibit zinc absorption. Finally, during Diet Period 3 (Zinc repletion period, d56-83), the subjects consumed a zinc adequate (11 mg Zn/day) diet for 28 days, with supplemental zinc (20 mg/d zinc as zinc gluconate) administered for the first 7 days. The subjects consumed a daily multiple vitamin supplements (100% RDA) without minerals (Long's Pharmacy, CA) throughout the study.

Diets

Adequate zinc (11 mg Zn/day) and low zinc (4 mg Zn/day) diets were initially calculated to provide 2500 kcal (55% of energy from carbohydrate, 15% from protein and 30% from fat). The actual amounts of energy that were offered were based on the Harris-Benedict equation for estimating basal energy expenditure (BEE) (184), multiplied by an activity factor of 1.5. Energy intakes were adjusted during the course of the study to maintain a constant body weight, by providing energy shakes devoid of zinc that were composed of non-dairy creamer, egg albumin, and flavor drink mix (Kool-Aid; Kraft Foods, Northfield, IL) in 200 kcal increments. Average total energy intakes ranged from 2461 to 3100 kcal (2838 ± 221 kcal, mean ± SD).

Adequate zinc diets contained beef as their main source of zinc and protein. Low zinc diets contained chicken as their main source of protein. Both diets were matched for protein amount from animal sources and had a phytate to zinc ratio of 10.

Macronutrient contents of study diets were estimated by Food Processor Pro software (ESHA Food Pro SQL ver 9.6, Salem, OR). Meals were served three times daily at 0830, 1200, and 1730. Salt and pepper seasoning packets were provided for addition to the meals ad libitum, and tap water was consumed ad libitum by subjects. Methylcellulose (2 to 4 g, Sigma Chemical, St. Louis, MO) was added to a daily beverage during Diet Periods 2 and 3 to ensure regular fecal flow, except during absorption study days.

A 4-day cycle of the adequate zinc diet and a five-day cycle of the low zinc diet were developed and reported previously (182). The diets were prepared by the dietetic staff of the metabolic kitchen at the UCSF GCRC of San Francisco General Hospital (SFGH) and the Ragle Human Nutrition Research Center at UC Davis.

Sample collection

Whole blood samples were collected into heparinized tubes by venipuncture of the fasting subjects on days 1, 13, 35, 55 and 83. An aliquot of whole blood was used for comet DNA damage analysis. Remaining whole blood was centrifuged at 1000 x g; plasma was removed by aspiration and frozen at -80° for further analysis. Erythrocytes were washed three times with cold saline solution, and frozen at -80° for further analysis.

Zinc and phytate analysis

Dietary zinc concentrations were measured with the use of inductively coupled plasma optical emission spectrophotometry (ICP-OES; Varian Vista Pro, Palo Alto, CA), and phytate concentrations in the study diets and test meals were measured by HPLC with the use of a Dionex Liquid Chromatograph System (Dionex Corp, Sunnyvale, CA) at the US Department of Agriculture, Agricultural Research Service at Cornell University, Ithaca, NY. Plasma and urine were divided into aliquots and stored at –20 ℃ until analyzed for zinc with ICP-OES (Varian Vista Pro).

DNA damage analysis

DNA single-strand breaks in peripheral blood cells were determined by alkali single-cell gel electrophoresis (comet assay) as described by Singh et al. (156).

Briefly, 25 µl fresh whole blood was mixed with 1 ml blood storage buffer (20 mM EDTA (ethylenediaminetetraacetic acid), 10% DMSO (dimethyl sulfoxide), HBSS (Hanks Balanced Salt Solution)) and centrifuged at 1000 rpm for 2 min to isolate blood cells. Blood cell pellets were mixed with 200 µl 0.5% Low Melting Point agarose and applied onto microscope slides (Trevigen, Gaithersburg, MD). Slides were stored in lysis buffer (Trevigen, Gaithersburg, MD) and shipped to Oregon State University within three days. At Oregon State University, cells embedded in the slides were lysed in comet assay lysis buffer containing 10% DMSO for 1 h. After lysis, DNA was allowed to unwind in alkali buffer (0.3 M NaOH, 1mM EDTA) for 20 min. Samples then underwent electrophoresis for 20 min at 300 mA and 25 V. After the electrophoresis, neutralization buffer (0.4M Tris, pH 7.5) was dropped onto slides to neutralize excess alkali buffer. The neutralized slides were immersed in cold 100% methanol, then 100% ethanol for 5 min each, and dried in air for storage. Immediately before measuring, nuclear material was stained by 20 µl/circle ethidium bromide (20µg/ml). Comet measurements were made by image analysis using Nikon E400 fluorescent microscope and Comet Assay III software. Images of 100 randomly selected nuclei (50 nuclei from each of two replicate slides) were blindly analyzed from each sample. The comet measurements of tail moment were recorded and used to indicate DNA damage in human peripheral blood cells. Inter-individual variation was minimized by using each individual as his own control and using a paired-analysis approach. We normalized tail moment from other time points to those from d13 (baseline), and statistical analysis was performed using fold change data for each individual relative to their own baseline.

Oxidative stress and antioxidant status

Plasma F_2-isoprostanes were measured as an index of lipid peroxidation and indicator of oxidative stress *in vivo* (185). The sum of various F_2-isoprostanes with the appropriate mass/charge ratio and fragmentation characteristics and arachidonic acid were measured in plasma, as described previously (157) with minor modification. Briefly, 500 µL plasma was subjected to alkaline hydrolysis, acidified, extracted with ethyl acetate-hexane, and dried under N_2 gas. The concentrated extract was separated by HPLC-MS/MS (High Performance Liquid Chromatography Shimadzu

HPLC system consisting of two LC-10ADvp pumps, a DGU-14A degasser, a SIL-HTC autosampler/system controller, and a CTO-10Avp column oven; Columbia, MD). The prostaglandin F (PGF) analytes and arachidonic acid were detected using selected reaction monitoring (SRM) on a triple-quadrupole mass spectrometer operated in negative mode (Applied Biosystems/MDS Sciex API 3000, Foster City, CA). Monitored in the PGF experiment were 15-series PGFs, *m/z* 353.2 to 193.1; 5-series PGFs, *m/z* 353.2 to 115.0; 8-iso-$PGF_{2\alpha}$-d4 internal standard, *m/z* 357.2 to 197.1. Standard curves were constructed using eight levels of the analytes 8-iso- $PGF_{2\alpha}$ and $PGF_{2\alpha}$ (100 to 4000 pg/mL). In the AA experiment, both AA and AA-d_8 internal standard (*m/z* 311.2 to 239.1) were monitored. Standard curves were calculated from 8 levels of AA (100 to 4000 ng/mL). Quantization was performed using the Analyst 1.4.1 software (Applied Biosystems/MDS Sciex). Inter-individual variation, for each subject was minimized by using each participant as his own control, and change in the isoprostane values relative to d13 (baseline) was calculated and analyzed with appropriate statistical analysis, as described below.

To assess plasma total antioxidant capacity, the ferritin reducing ability of plasma (FRAP) was measured as described (158). Briefly, diluted plasma samples (1:4) were mixed on a 96-well plate with 300 μL freshly prepared FRAP reagent (25 mL sodium acetate buffer (300 mmol/L), 2.5 mL 2,4,6-tripyridyl-1,3,5-triazine (TPTZ, 10 mmol/L), and 2.5 mL $FeCl_3$ (20 mmol/L)). Samples were incubated for 15 min at 37℃ before reading at 593 nm on a microplate reader (Spectramax 190; Molecular Devices, Sunnyvale, CA). FRAP values were calculated using trolox as standards.

Plasma α-tocopherol and γ-tocopherol were extracted and measured by HPLC-ECD (high performance liquid chromatography-electrochemical detection) as described previously (159, 160). Concentrations of α-tocopherol standards were determined spectrophotometrically using $\varepsilon 292\ nm^{EtOH} = 3270\ M^{-1} \cdot cm^{-1}$ and $\varepsilon 298\ nm^{EtOH} = 3810\ M^{-1} \cdot cm^{-1}$ for γ-tocopherol (159).

Superoxide dismutase (SOD) activity was determined by the xanthine oxidase-cytochrome c method according to McCord and Fridovich (162) and L'Abbe et al (163). Hemoglobin concentration in cell lysate was determined by Drabkin's method (164). Briefly, the reaction was initiated by adding xanthine oxidase (Calbiochem, CA) into the reaction mix containing 20 mM sodium carbonate buffer (pH 10.0), 0.1 mM

EDTA, 50 μM xanthine (Sigma), and 10 μM cytochrome c (Sigma) in a final volume of 200 μl. The rate of increase in absorbance at 408 nm was monitored by plate reader (Molecular Device, CA). One SOD activity unit was defined as the quantity of SOD required to produce 50% inhibition of the reduction rate of cytochrome c under the experimental conditions.

Sample size and statistical analyses

Based on the actual sample size of 9 men (2 subjects missing d13 comet slides) and the observed variation in tail moment, we were able to detect a within-subject difference in tail moment of 0.51 by dietary zinc periods, with 80% power and a 5% chance of type I error.

Statistical analysis was performed with the use of PRISM (version 4.0; GraphPad Software, San Diego). Comparisons between time points were made by Repeated Measures ANOVA with Bonferroni's Multiple Comparison post-hoc test when appropriate. Multiple regressions were conducted to assess the association of change between plasma zinc and DNA strand breaks with subjects as a factor. Data were considered statistically significant at $P<0.05$. All data are reported as mean ± SEM unless otherwise indicated.

4.4 Results

The subject characteristics and zinc status measurements throughout the study are published elsewhere (182). In summary, the subjects' mean (±SD) age was 38 ± 8 years and their BMI ranged from 20.8-25.9. None of the subjects were anemic. Mean ± SD dietary zinc intake and plasma zinc concentrations were 12.9 ± 2.2 mg/d and 0.85 ± 0.08 μg/mL, respectively, at the start of the study. The comet assay has been used in human intervention trials to measure DNA single strand breaks *in vivo* from peripheral blood cells (186). Thus, we used this technique to evaluate the effects of dietary zinc intake on DNA integrity. During the zinc depletion period (DP2), DNA strand breaks increased, as indicated by increased average tail moment (**Figure 4.2**). In particular, the average tail moments increased 57% at the end of the depletion period, in comparison to the baseline (d13 vs. d55, $P<0.05$) suggesting that 6 weeks

of low zinc intake significantly increased DNA damage in mixed peripheral blood cells. Importantly, the increases in DNA damage were reversible following zinc repletion, suggesting that the extent of DNA damage was dependent upon dietary zinc status. At the end of the zinc repletion period (DP3), the average tail moments were reduced 39.9% in comparison to the end of the depletion period (d83 vs d55, $P<0.01$), and were about 91.8% of the baseline level (d83 vs d13, $P>0.05$), demonstrating that zinc repletion normalized DNA damage. Interestingly, we detected a 34% trend for decrease in average tail moments during the baseline period (DP1, d13 vs d1, $P=0.09$) suggesting that the subjects may have had moderate DNA damage before the enrollment into the study. Collectively, these data suggest that consuming a zinc-adequate balanced-nutritious diet (11 mg Zn/day) for 13 d may have helped to reduce DNA damage thereby lowering "baseline" levels to below those observed initially, when the volunteers were still consuming their usual diets. Altogether, these data suggest that dietary zinc status affects DNA damage in peripheral blood cells and that adequate zinc status may be essential to maintain DNA integrity in humans. Importantly, the alterations in DNA integrity occurred before significant changes in plasma zinc were detected. During the study, as reported previously, plasma & urinary zinc concentrations measured at the beginning (d13) and the end of the zinc depletion period (d55) did not differ. Mean plasma zinc concentrations were 0.79 ± 0.9 μg/mL and 0.79 ± 1.0 μg/mL on day 13 and day 55, respectively. However, plasma zinc concentrations were 13% higher at the end of the zinc repletion period compared to the end of zinc depletion period (0.86 ± 1.0 μg/mL; day 83 vs day56, $P<0.05$). Urinary zinc concentrations were not significantly different between metabolic periods (182). Although there was no statistical change in plasma zinc concentrations during zinc depletion period, plasma zinc concentrations show a negative correlation with DNA damage parameters (**Figure 4.3A**). Interestingly, the correlations were greater during the zinc depletion/repletion period of the study, and the correlation during the baseline period was not statistically significant (**Figure 4.3B&C**).

We measured plasma total antioxidant capacity by FRAP assay, which was unaffected by zinc intake throughout the investigation (**Table 4.1,** $P=0.37$). However, plasma concentrations of α-tocopherol and γ-tocopherol concentrations differed

significantly by dietary period (Table 4.1, α-tocopherol, *P*=0.004; γ-tocopherol, *P* = 0.0013). Specifically, tocopherol levels tended to increase during the zinc depletion period (d55 vs d13, α-tocopherol increased 14%, *P*=0.30; and γ-tocopherol increased 26%, *P*=0.07). During zinc repletion γ-tocopherol levels decreased significantly, whereas α-tocopherol levels did not change (d83 vs d55, α-tocopherol decreased 8%, *P*>0.05; γ-tocopherol decreased 30%, *P*<0.05).

We also measured erythrocyte superoxide dismutase (SOD) activity as an additional component of the antioxidant defense system (187). CuZnSOD is the only SOD in the erythrocyte, and it is an important zinc-containing antioxidant defense enzyme found in the circulation. Erythrocyte SOD activity tended to decrease after marginal zinc depletion and increase after zinc repletion, but the changes was not statistically significant (Table 4.1).

To determine whether zinc depletion affected lipid peroxidation and oxidative stress, we measured plasma F_2-isoprostane concentrations. F_2-isoprostanes are generated from non-enzymatic peroxidation of arachidonic acid and are considered the reference standard for the measurement of lipid peroxidation and oxidative stress (185). Concentrations of F_2-isoprostanes in biological fluids are highly related to the incidence of many oxidative stress-associated diseases, such as diabetes, obesity and cardiovascular diseases (188). Arachidonic acid (AA) is the precursor fatty acid of F_2-isoprostanes and we hypothesized that plasma AA could be affected by changes in altered zinc status (15). Thus, we measured plasma AA concentrations simultaneously with F_2-IsoPs, but found that plasma AA concentrations did not change significantly during the study (Table 1, P =0.26). Likewise, dietary zinc depletion did not affect plasma . 8-iso-PGF2α and 5 series F_2-isoprostanes concentrations (**Figure 4.4**). Similar to the observations concerning DNA single-strand breaks, however, F_2-isoprostanes tended to decline during the baseline period (d1 vs d13). The average (mean ± SE) F_2-isoprostanes concentrations on d13 after the baseline period were 2131±835.7 pg/ml for 8-iso-PGF2α and 4323±1187 pg/ml for 5 series F_2-isoprostanes..

4.5 Discussion

The present intervention study shows that, low dietary zinc intake resulted in an increase in DNA single-strand breaks in healthy human volunteers. Consumption of a marginally zinc deficient diet by these same subjects had no significant effect on their plasma and urinary zinc levels (182) which are commonly used biomarkers for assessing zinc status. However, plasma zinc concentrations were correlated with changes in DNA strand breaks during dietary zinc/repletion. It is difficult to assess an individual's zinc status because the symptoms of zinc deficiency are usually nonspecific, and homeostatic mechanisms tightly maintain tissue and circulating zinc levels within fairly narrow ranges. The existing biomarkers for zinc deficiency, such as plasma zinc concentrations and activities of zinc-dependent enzymes, are problematic in that they lack sensitivity and/or specificity for individual-level assessment (189). The poor sensitivity and specificity of plasma zinc hinder the progress of research on zinc deficiency and its potential health consequences. We report here, using a highly controlled human zinc feeding trial, those alterations in DNA integrity occur quickly with marginal zinc depletion; and, importantly, these changes occur prior to measurable alterations in plasma zinc levels. Our data support the notion that functional consequences of zinc deficiency occur before plasma zinc concentrations decline, and highlight the essential role of zinc for maintaining DNA integrity *in vivo*.

Previous studies, using *in vitro* and *in vivo* animal models, found that zinc deficiency increased DNA damage in liver or testes (57, 58, 60, 61). To our knowledge, our study is the first report of zinc-related DNA damage in peripheral blood cells of humans. Although previous published and the current studies did not specify the differences of sensitivity to DNA damage among tissues with zinc depletion, our lab observed that marginal zinc deficiency increased DNA strand breaks in peripheral blood cells, without significant increasing in 8-oxodG levels in tissues such as the liver or prostate (unpublished, JN paper in revision). Increases in DNA damage with marginal deficiency in tissues are only seen with additional exogenous stresses (unpublished data), suggesting that the peripheral blood cells may be more sensitive to DNA damage with zinc deficiency.

Possible links between zinc deficiency and DNA damage were described previously, but the specific underlying mechanisms for these events remain unknown. Possible mechanisms include perturbations of antioxidant defenses and/or impaired DNA repair mechanisms. We have shown in previous in vitro studies that cellular zinc depletion causes oxidative stress and disruptions of DNA repair pathways (57) and further investigation into the effects of human zinc depletion on DNA repair is an important area of future research. In the current study, no changes in total antioxidant capacity were observed with marginal zinc depletion, although we did see a trend for decreased erythrocyte SOD activity. This result is consistent with one study in humans by the European Zincage project (18), which reported that erythrocyte SOD activity was inversely associated with the plasma zinc concentration.

We have previously shown an increase in plasma F_2-isoprostanes in zinc deficient rats, indicating that lipid peroxidation increased with zinc depletion (152). In marginally zinc deficient rats we also found that hepatic vitamin E status was compromised with zinc deficiency, while plasma vitamin E concentration remained unchanged (submitted manuscript), results which are consistent with our current findings. Therefore, we postulated that during zinc-depletion, liver vitamin E status is sacrificed in order to maintain circulating vitamin E concentrations. In marginal zinc depletion in humans, we did not detect any changes of F_2-isoprostanes or plasma tocopherol concentrations during zinc depletion and repletion, which could be due to similar compensatory mechanisms as shown in our previous rat studies. The body may be able to partially suppress lipid peroxidation caused by zinc deficiency by maintaining plasma vitamin E concentrations. Importantly, although compensatory antioxidant metabolism may help limit oxidative stress caused by low cellular zinc, this was not sufficient to prevent the DNA damage to peripheral blood cells, suggesting that oxidative stress may not be the only factor contributing to DNA damage with zinc deficiency.

One of the interesting findings from the current study is that both DNA single-strand breaks and F_2-isoprostane measures tended to be elevated upon entry into the study (d1). The average (mean $\pm$ SE) tail moment after baseline period was 1.008 ± 0.075, while at entry to the study the mean comet tail moments was 1.898 ± 0.260. While comparison of comet values between studies is problematic due to a lack of

standardization of the assay between labs, the values seen at the end of our baseline period (d13) are close to the healthy control comet moment values (~ 1.0) that were reported in studies that used similar methodology and analysis (190-192). Several studies have shown a positive correlation between DNA damage/comet moment and heavy smoking. Heavy smokers were found to have tail moments in their peripheral blood of 1.5 ± 0.29, while healthy controls were 1.00±0.016 (191). The elevated comet measurements on day 0 in the current study imply that although the entry criteria were designed to exclude potentially zinc-deficient individuals, the subjects may have had suboptimal zinc or other micronutrient status upon enrollment into the study, which was reversed during the baseline period. The subjects were also given a multivitamin supplement (including 30 IU *all-rac* α-tocopherol.), so we may have also corrected other micronutrient deficiencies during the baseline period. Several other vitamins, such as vitamins E, C and folate have antioxidant and/or DNA integrity functions (193). This finding during the baseline period also highlights the potential sensitivity of DNA damage to micronutrient depletion. However, it is also important to point out that subjects remained on multivitamin supplements throughout the study, and correlations with plasma zinc and DNA damage were only significant during the zinc depletion/repletion phases. Thus, alterations in oxidative stress parameters and DNA damage after baseline period should be largely attributed to zinc alterations.

The comet assay has become a widely used assay in the toxicology field; and it is used as a quick, sensitive and relatively inexpensive method for measuring DNA strand breaks as an index of genetic damage following exposure to genotoxic agents. A similar approach may be taken for monitoring the status of micronutrients, such as zinc, that play a critical role in DNA integrity. However, results of the comet assay are not specific to zinc status, and these results may be affected by other environmental stresses, including other micronutrient deficiencies (193, 194). In addition, further standardization of the assay needs to be performed to control for inter-laboratory variations. Currently in the genotoxicity community, there is a large push to establish standardized techniques, establish reference values/internal standards and to standardize statistical analysis for the assay (195-197). Altogether, the high sensitivity of measuring DNA strand breaks by comet assay may complement the poor sensitivity of plasma zinc concentration, and help identify individuals with marginal

zinc deficiency and test the effectiveness of treatments. For example, supplementing zinc in high risk populations, such as the elderly or vegetarians, for a few weeks may not change plasma zinc levels or improve any clinical symptoms; but decreases in DNA strand breaks, as measured by comet assay during the supplementation period, could imply a preexisting marginal zinc deficient status and justify the continuation of supplemental zinc. Moreover, other functional assays such as measuring IL-2 gene expression in mononuclear cells with in vitro zinc addition could be a good assay for detecting human marginal zinc deficiency (198).

The prevalence of zinc-deficiency in developed counties could be underestimated. Data from NHANES (2001-2002) shows that approximately 12% Americans do not consume enough zinc relative to their theoretical requirements. The current dietary intervention study shows that dietary zinc-depletion increases DNA damage in humans, suggesting that marginal zinc deficiency could have significant health consequences due to zinc's essential role in maintaining DNA integrity. Moreover, the current study highlights the sensitivity of DNA integrity to marginal dietary zinc depletion compared to traditional zinc status measures and indicates that DNA single-strand breaks in peripheral blood cells could be used as a complementary biomarker for zinc deficiency in humans, which may help the progress of clinical diagnosis and zinc-deficiency research.

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The author's responsibilities were as follows---YS: managed all aspects of studies done in Oregon State University, including conducting and design all experiments, data interpretation, and initial draft of the manuscript; CSC: managed all aspects of the original clinical studies; RSB: conducted the FRAP assay; MGT: contribute to the data interpretation and manuscript revision; KHB, JCK and EH: contributed to the study concept, research design, data interpretation, and manuscript

revision. All authors critically reviewed the manuscript. None of the authors had a personal or financial conflict of interest.

Table 4.1 Antioxidant status of human subjects during zinc depletion and repletion

	d1	d13	d35	d55	d83	*P*
FRAP (μmol/L)	613.5± 31.3[a]	606.5± 23.7[a]	636.6± 30.2[a]	634.8± 30.01[a]	594.4± 36.8[a]	0.37
Plasma α-tocopherol (μmol/L)	20.49± 1.61[a]	23.67± 1.98[a,b]	26.30± 1.73[b]	27.13± 1.78[b]	25.20± 1.75[b]	0.004
Plasma γ-tocopherol (μmol/L)	1.55±0.23[a]	1.69± 0.18[ab]	2.10±0.14[b]	2.13±0.22[b]	1.48±0.13[a]	0.0013
Erythrocyte SOD activity (U/mg Hb)	99.5±4.5[a]	96.1±2.0[ab]	89.2±4.9 [b]	89.2±3.7 [b]	91.3±3.6 [b]	0.001
Arachidonic acid (μg/ml)	147.0± 13.8[a]	141.4± 13.7[a]	135.6± 12.4[a]	138.0± 14.5[a]	139.3± 11.7[a]	0.37

Significant differences between means are determined by repeated-measures analysis of variance. The P values shown in the table are the main effects of the analysis. Values (means ± SEM, n=9) across a row with different superscript letters are significantly different (P<0.05) as determined by Bonferroni's Multiple Comparison test.

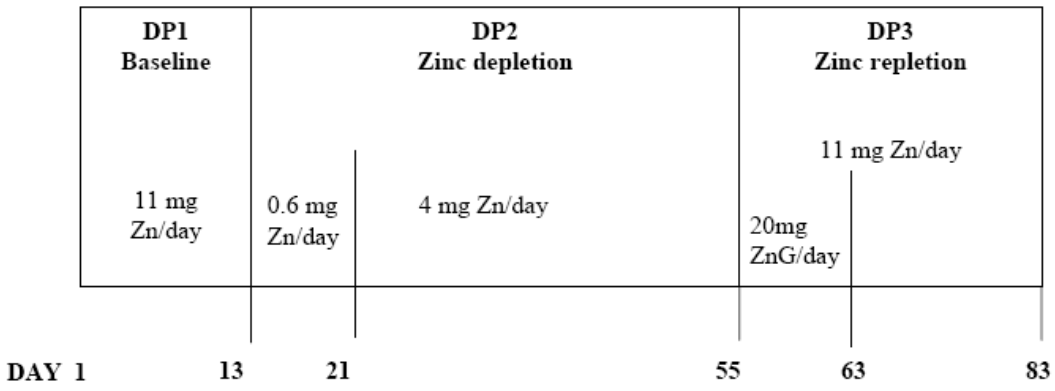

Figure 4.1 Study design of the dietary intervention study. The figure is adapted from the previously reported paper (182). The study was divided into 3 Dietary Periods (DPs). From d1 to d13 was baseline diet period (DP1) during which zinc adequate diet (11 mg Zn/day) was given to human subjects to ensure zinc adequate status. From d14 to d55 was zinc-depletion diet period (DP2) during which subjects were given a liquid diet (0.6 mg Zn/day) for 7 days followed by a low-zinc diet (4 mg Zn/day) for another 35 days. Phytate (1.3 g/day) was added to the diet from d14 to d34. From d56 to d83 was zinc repletion period (DP3) during which subjects were give zinc-adequate diet (11mg Zn/day) with additional supplemental zinc (20 mg Zn/day as zinc gluconate, ZnG) given for the first 7 days.

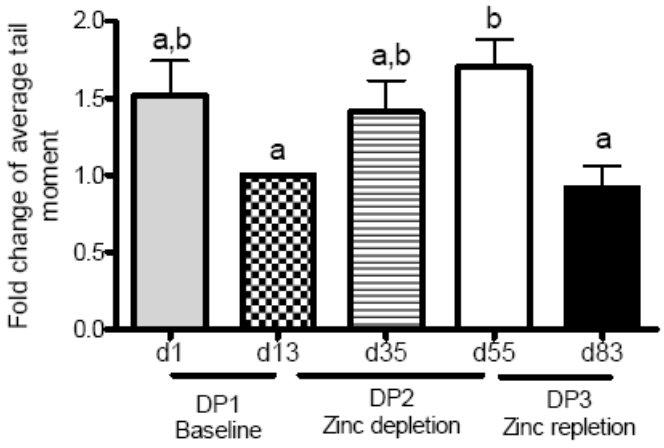

Figure 4.2 Six weeks of dietary zinc depletion increased DNA single-strand breaks in human peripheral blood cells. DNA single-strand breaks were measured by comet assay as described in *Sample collection and analysis*. Blood samples were collected on d1 (beginning of the study), d13 (baseline), d35 (middle of zinc depletion), d55 (end of zinc depletion) and d83 (end of zinc repletion). Two comet slides were made for each human subject at each time point. Image of 100 randomly selected nuclei (50 nuclei from each of two replicate slides) were measured blindly for tail moment. Results are presented as mean fold change of average tail moments to those from d13 (baseline) for each subjects. Values are mean ± SEM (n = 7). Significant differences between means were determined by Repeated Measures ANOVA followed by Bonferroni's Multiple Comparison test. Significant differences between means were determined by Repeated Measures ANOVA followed by Bonferroni's Multiple Comparison test. Groups with different letters are significantly different from each other. DNA damage gradually increased during zinc depletion, and zinc repletion reversed the damage back to baseline levels.

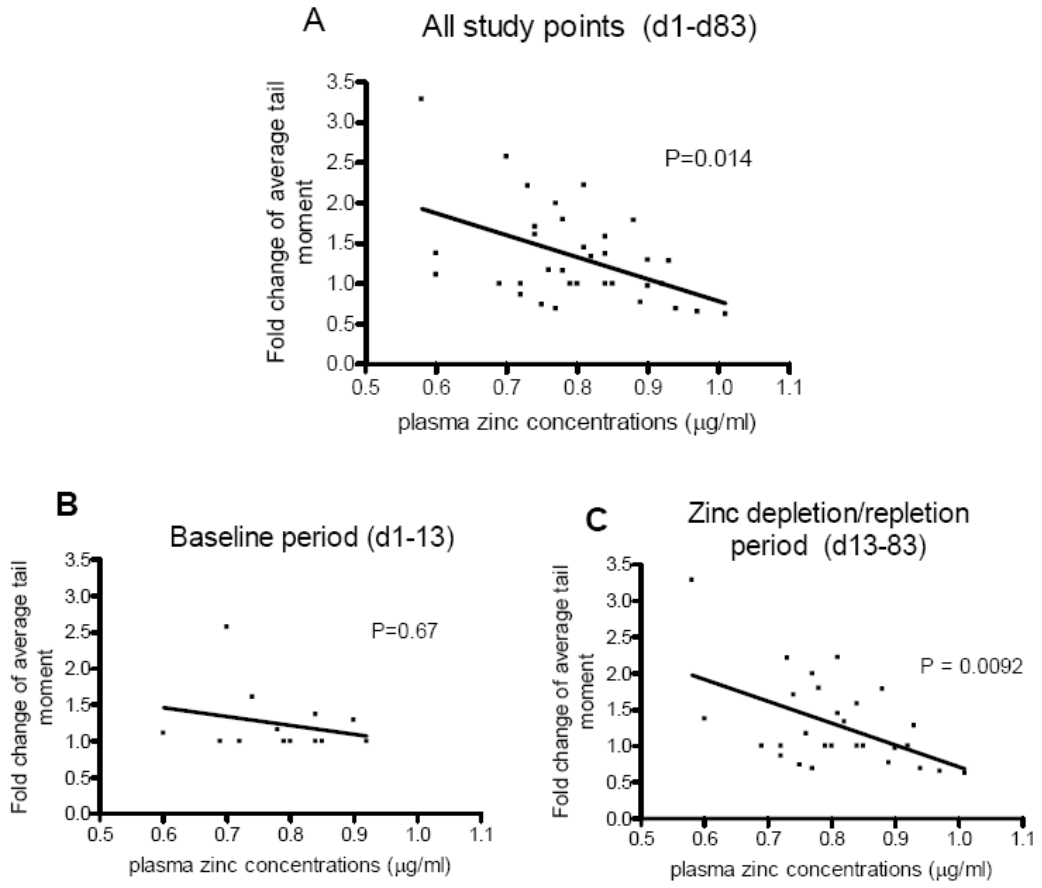

Figure 4.3 DNA strand breaks in peripheral blood cells are significantly correlated to plasma zinc concentrations. Fold changes of average tail moments in peripheral blood cells are plotted as a function of plasma zinc concentrations, with P-value of the correlation coefficient, during (A) d1-d83, whole study period (B) d1-d13, baseline period and (C) d13-d83, zinc depletion/repletion period. Multiple regression were conducted to assess the association of change between variables with subjects as a factor. DNA single-strand breaks were measured by comet assay and plasma zinc concentrations is measured by ICP-OES as described in Sample collection and analysis. Blood samples were collected on d1 (beginning of the study), d13 (baseline), d35 (middle of zinc depletion), d55 (end of zinc depletion) and d83 (end of zinc repletion). Two comet slides were made for each human subject at each time point. Image of 100 randomly selected nuclei (50 nuclei from each of two replicate slides) were measured blindly for tail moment. Results are presented as mean fold change of average tail moments to those from d13 (baseline) for each subjects. There was a significant correlation between fold change of average tail moments in peripheral blood cells and plasma zinc concentrations for the whole study period (P=0.0052) and zinc depletion/repletion period (P=0.0063), but no significant correlation for the baseline period. (P=0.42).

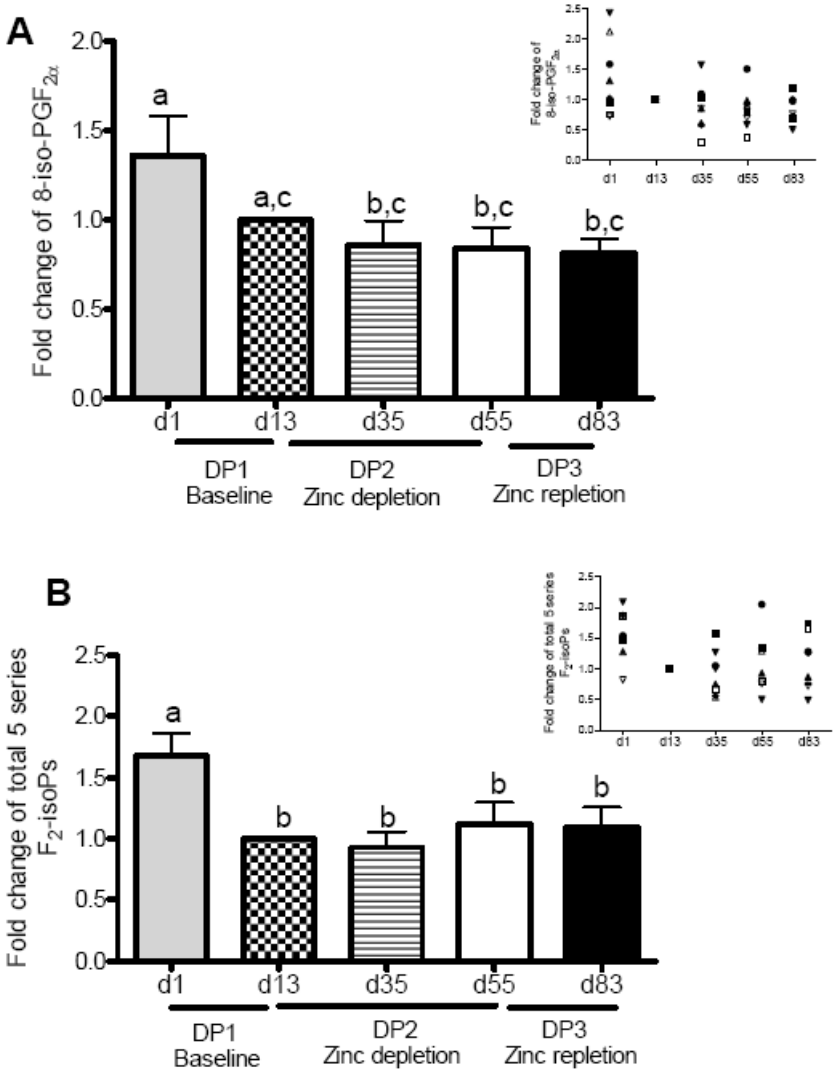

Figure 4.4 Dietary zinc depletion did not change plasma F_2-isoprostanes. (A) 8-iso-$PGF_{2\alpha}$, data during zinc depletion and repletion, (inset individual subject data). Values calculated based on the sum of 8-iso-15(R)-$PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$ (pg/ml) and percent change relative to D13 baseline; (B) total 5-series F_2-isoprostanes data during zinc depletion and repletion (inset individual subject data). All isoprostanes were measured as described in *Sample collection and analysis.* Values are mean ± SEM (n = 8). Significant differences between means were determined by Repeated Measures ANOVA followed by Bonferroni's Multiple Comparison test. Groups with different letters are significantly different from each other ($P < 0.05$).

Chapter 5 Expression and regulation of zinc transporter profiles to maintain prostate zinc homeostasis in rats

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5.1 Abstract

Zinc plays important roles in numerous cellular activities and physiological functions. Since there is little storage of zinc in the body, intracellular zinc levels are strictly maintained by zinc homeostatic mechanisms. Zinc concentrations in the prostate are the highest of all soft tissues and could be important for prostate health. However the mechanisms by which the prostate maintains high zinc levels are still unclear. In addition, the response of the prostate to alterations in dietary zinc is unknown. The current study explored cellular zinc levels and zinc transporter expression profiles in the lobes of the prostate during dietary marginal zinc depletion. Rats were given either zinc-adequate (ZA, 30mg Zn/kg) or marginal zinc-deficient (MZD, 5mg Zn/kg) diet for 9 weeks. In addition, a subgroup of the MZD rats was supplemented with phytase (1500 unit/kg diet) to improve zinc absorption. We found that both zinc concentrations and ZnT2 expression in the prostate dorsolateral lobes were substantially higher than in the ventral lobes. Marginal zinc depletion significantly decreased ZnT2 expression in the dorsolateral lobes ($P<0.05$), and phytase supplementation had a trend to increase ZnT2 expression. No correlations were found between zinc transporter expression and zinc concentrations in the ventral lobes. In addition, of all measured zinc transporters, only ZnT2 mRNA abundance was significantly correlated to the zinc concentrations in the dorsolateral lobe. These results indicate that ZnT2 may play a major role in the maintenance of zinc homeostasis in the prostate.

5.2 Introduction

Zinc is an essential trace mineral and is ubiquitously distributed in hundreds of proteins in mammalian cells. Since zinc is widely involved in the normal functions of many proteins, zinc is required for a variety of biological activities such as growth & development, immune response, wound healing, neurological function and reproduction (1). Intracellular zinc levels are strictly controlled by zinc homeostatic mechanisms that maintain stable zinc supply and appropriate zinc distribution in cells. The activities of zinc uptake, efflux and cellular compartmentalizing rely on the functions of zinc transporters. Two zinc transporter gene families have been identified in mammals: the solute-linked carrier 30A (SLC30A, ZnT) family and the SLC39A (Zip) family of metal ion transporters. The ZnT family of protein functions in zinc efflux from cytoplasm to either extracellular space and/or intracellular vesicles. The ZIP family of protein functions in zinc uptake from the extracellular matrix and/or intracellular organelles into the cytoplasm (117). These zinc transporters are expressed in a tissue-specific manner, and respond differentially to dietary zinc levels and physiological conditions. Therefore, it has been proposed that a loss of function or dysregulation of certain zinc transporters would result in an impairment of zinc homeostasis and predispose the body to zinc-imbalance-related diseases, such as cancer, asthma, diabetes, and Alzheimer's disease (141).

The maintenance of zinc homeostasis in the prostate may be even more critical than the other organs. The prostate contains the highest zinc concentration of any other soft tissues in the body. The prostate epithelial cells have a characteristic high aerobic glycolytic activity, low respiration (102-104) and high citrate secretion. Zinc may be required for maintaining these properties by reducing the activity of mitochondrial aconitase and inhibiting the terminal oxidation through the electron transport chain (105, 106). Moreover, as the prostate becomes malignant, prostate zinc concentration decreases by 75-90% (93). Therefore, it has been proposed that maintaining the high zinc content is essential for prostate health, and loss the ability to accumulate high zinc levels may contribute to the prostate malignancies (94-98). However, little is known about mechanisms by which the normal prostate maintains high levels of zinc. Therefore, the objectives of the current study were to examine the

mechanisms by which the prostate modulates zinc concentrations and profile zinc transporter expression during dietary marginal zinc depletion.

The current study is one of the first to quantitatively evaluate the correlations between zinc transporter expressions and zinc concentration in the prostate with dietary zinc depletion. The results from this study provide insights into the mechanisms by which the prostate regulates cellular zinc levels and provide directions for future studies establishing connections between defects in zinc transporter regulation and the development of prostate diseases.

5.3 Material and Methods

Animals Male Sprague-Dawley rats (n=12, 4 wk old, 125-150 g) from Charles River Laboratories, Inc. (Wilmington, MA), were housed individually in polycarbonate cages and acclimated for 2 wk to the temperature- and humidity-controlled environment with a 12-h-dark: light cycle. Rats were fed a zinc-adequate (ZA, 30mg Zn/kg) or marginal zinc-deficient (MZD, 5-6mg Zn/kg) diet for 9 weeks. To further examine the effects of phytase on zinc status and DNA integrity in the prostate, 1,500 phytase units/kg was added to the MZD diets and fed to the rats (MZD+P). Diets were based on modified AIN-93M diets (154) formulated with egg white rather than casein and with zinc provided as zinc carbonate (Dyets, Bethlehem, PA). Deionized water was provided as drinking water. Rats were killed following anesthesia with isoflurane overdose (1-5%; Henry Schein; Melville, NY). Diet intakes and body weights were measured twice per week. This study was approved by the Institutional Animal Care and Use Committee (IACUC) at USARIEM and Oregon State University, and animals were maintained in accordance with IACUC's guidelines for the care and use of laboratory animals.

Tissue and blood collection Blood samples were collected by cardiac puncture into trace element-free vials containing EDTA. Plasma was separated immediately and frozen at -80ºC until analysis. Samples of rat prostate dorsolateral and ventral lobes were dissected and immediately half snap frozen at -80℃ and half stored in RNA stabilization solution (RNAlater, Ambion, Austin, TX) until further analysis.

Zinc analysis Plasma (n=8), ventral lobe (n=6) and dorsolateral lobe (n=5) zinc concentrations were determined by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES; Teledyne Leeman Labs, Hudson, New Hampshire) with small modification of a previous described method (155). Samples were digested in 1ml 69%-70% OmniTrace nitric acid (VWR, West Chester, VA) overnight. Following digestion, samples were diluted 10 times with water treated with chelex 100 resin (Bio-rad, Hercules, CA) and analyzed by ICP-OES against known standards (152).

Quantitative Real-time PCR analysis (qRT-PCR) The mRNA abundance of zinc transporters in rat prostates (ZnT1-4 and Zip1-4) were measured by qRT-PCR. RNA was extracted from prostates using a RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). First-strand cDNA was reverse transcribed from the isolated RNA using Superscript First-Strand Synthesis System according to the manufacturer's instructions (Invitrogen Corporation, CA). Primers and annealing temperatures of each transporter gene are listed in **Table 5.1.** Real time PCR reactions were performed using DyNAmo HS SYBR Green qPCR kit (New England Biolabs, Ipswich, MA). Gene copies were determined using the standard curve method. A standard curve was generated from serial dilutions of purified plasmid DNA that encoded for the gene of interest. Data represent averaged copy number normalized to the 18s housekeeping gene.

Statistical analysis Statistical analysis was performed with the use of PRISM (version 4.0; GraphPad Software, San Diego, CA). Significantly differences between means were analyzed by one-way ANOVA followed by Bonferroni post-hoc test when appropriate. Pearson correlation coefficients were calculated between zinc transporter expression and zinc concentrations. Appropriate data transformations were taken when uneven variations were detected. Differences were considered statistically significant at $P<0.05$. All data are reported as means ± SEM (standard error of the mean) unless otherwise indicated.

5.4 Results

There were no changes in food intakes among dietary groups (data not shown, $P>0.05$), but the body weights in the MZD group (312.8 ± 5.3 g) were significantly lower than in the ZA group (368.7 ± 6.8 g, $P<0.001$). Phytase supplementation significantly increased body weights in the rats (346.2±5.5 g, $P<0.05$).

Zinc concentrations in the MZD group decreased 44% in plasma and 71% in the prostate dorsolateral lobes compared to the ZA rats (**Table 5.2,** $P<0.05$). In contrast, zinc concentrations in prostate ventral lobes were not affected by marginal zinc depletion, indicating that prostate dorsolateral lobes were more susceptible to marginal zinc depletion. In addition, phytase supplementation significantly increased plasma and dorsolateral lobe zinc concentrations (Table 2, $P<0.05$), indicating that phytase helped improve zinc status in the rats fed the MZD diet.

Prostate dorsolateral lobes contain higher zinc concentrations than the ventral lobes, which was also observed in the current study. Zinc transporter mRNA expression profiles of the ZA rats were compared between the dorsolateral and ventral lobes. The mRNA levels of ZnT3, Zip2 and Zip4 in both lobes were very low in comparison with the other transporters (**Figure 5.1**). No significant differences in ZnT1, ZnT4, Zip1 and Zip3 mRNA levels were detected between the dorsolateral and ventral lobes. However, ZnT2 gene expression levels were markedly different between the dorsolateral and ventral lobes. The mRNA abundance of ZnT2 in the dorsolateral lobes was about 1000 times higher than that in the ventral lobes ($P<0.05$), indicating that ZnT2 may play an important role in accumulating very high levels of zinc in rat prostate dorsolateral lobes.

Correlations between zinc concentrations and zinc transporter mRNA levels in the dorsolateral lobes of all rats in all dietary groups were analyzed by Pearson analysis. We found that only ZnT2 mRNA levels were significantly correlated to the alterations of zinc concentrations in the dorsolateral lobes ($r=0.38$, $P=0.034$, **Table 5.3**). We evaluated the effects of dietary zinc intake on ZnT2 expression, and found that marginal zinc depletion significantly decreased ZnT2 mRNA levels specifically in the prostate dorsolateral lobes (**Figure 5.2** $P<0.05$) and phytase supplementation partially restored ZnT2 mRNA levels. No effects on ZnT2 or other zinc transporters were detected in the ventral lobes. Altogether, these results indicate that ZnT2 may

play an important role in maintaining dorsolateral lobe zinc homeostasis during dietary zinc depletion.

5.5 Discussion

The current study shows that the dorsolateral lobes had higher ZnT2 mRNA levels as well as higher zinc concentrations than the ventral lobes of the prostate. ZnT2 mRNA levels in the dorsolateral lobes were also significantly correlated to dorsolateral zinc concentrations, and ZnT2 decreased with marginal zinc deficiency. These results indicate that ZnT2 may play an essential role in modulating prostate zinc homeostasis. Altogether, the current study gives insights for understanding the mechanisms in place that maintain prostate zinc homeostasis, and helps identify zinc transporters that may play roles in zinc regulation.

The prostate contains high levels of zinc, and zinc may play an essential role in the prostate. Prostate epithelial cells have characteristicly high aerobic glycolysis and low respiration rates (102-104), and zinc may be required for these properties via inhibition of aconitase. Costello et al found that zinc decreases the activity of mitochondrial aconitase and inhibits the terminal oxidation through electron transport chain, thus contributing to the properties of high citrate secretion and low respiration in the prostate (105, 106). Therefore, a loss of zinc in the prostate may remove the inhibitory effects of zinc on mitochondrial terminal oxidation, and increase respiration rates and possibly increasing the production of free radicals by the electron transport chain. In our laboratory, we have also postulated an important function of zinc in the prostate may be maintaining DNA integrity by reducing oxidative stress and preserving DNA repair functions (142). We found that loss of cellular zinc levels in the prostate epithelial cells resulted in oxidative stress and increased DNA damage (70).

Since high zinc concentrations may be essential for prostate health, a failure to maintain zinc homeostasis may increase the risk of prostate cancer. Dysregulation of zinc transporters including ZnT1, ZnT3, Zip1, Zip2 and Zip3 have been found to be associated with the low intracellular zinc content in human prostate cancer tissues or prostate epithelial cancer cell lines. *hZIP1, hZIP2 and hZIP3* gene and/or protein expressions were downregulated in human prostate adenocarcinomatous glands and

malignant cell lines (98). In addition, tumorigenic human prostate epithelial cell lines (RWPE2) had decreased ZIP1 protein expression and redistributed intracellular ZIP3 in comparison with the non-tumorigenic human prostate epithelial cell line RWPE1 (95). Moreover, overexpression of *hZIP1* in the tumorgenic RWPE2 prostate cells increased the intracellular zinc concentrations, induced cell apoptosis and suppressed cell growth (95). Normal prostate tissues from African American men had higher expressions of *ZIP1* and *ZIP2* than the tissues from the Caucasin men, which was associated with a higher incidence of prostate cancer in African Americans (94). In terms of the ZnT family members, lower levels of ZnT1 gene are expressed in human prostate cancer tissues (144) and in the androgen-independent subline of LNCaP cells(145). ZnT4 gene expressions are also decreased in human prostate benign hyperplasia (BPH) and carcinoma tissues (146). These previous studies provide important preliminary evidence for the potential role of specific zinc transporters in prostate cancer progression.

The current study quantitatively evaluated zinc transporter mRNA expressions in normal rat prostates. We found that ZnT2 was the only zinc transporter whose mRNA levels were strongly correlated with the zinc concentrations in the dorsolateral lobes ($P<0.03$) and were reduced by marginal zinc depletion. However, similar effects are not observed in every tissue. For example, erythrocyte ZnT1 was reduced and Zip10 was increased by dietary zinc depletion in mice (199), cDNA microarray in human THP-1 mononuclear cells revealed an increase in ZnT1 and ZnT7 with zinc supplementation and an increase in Zip2 with zinc depletion (128). In the brain, ZnT1 was reduced by dietary zinc depletion in rats (200), and dietary zinc depletion increased Zip4 (134)and decreased ZnT1 and ZnT2 (112) expression in rodent small intestines. Different zinc transporters are expressed and regulated by dietary zinc intake in different tissues and cells, suggesting that the regulation of zinc transporters is rather tissue specific.

The primary function of ZnT2 may be to transport zinc from the cytoplasm into endosome or lysosomes (115). Thus it is possible that a decrease in ZnT2 expression with marginal zinc deficiency in the prostate may reduce the amount of zinc transported into endosomes or lysosomes. In that case, zinc may be preserved for other cell compartments, such as the mitochondria and nucleus, and may be more

available for activities more essential for prostate cell function and survival. Future studies to understand zinc homeostatic mechanisms in the prostate should focus on the redistribution of subcellular zinc levels during zinc depletion and identify cellular compartments which are most resistant to zinc depletion.

Although zinc concentrations are high in the whole prostate, zinc is not uniformly distributed among all prostate lobes. The human prostates are composed of the peripheral zone (70%), the central zone (25%) and the transition zone (5%). The peripheral zone contains much higher zinc and citrate than the other zones, and 80% of prostate malignancies develop in the peripheral zone (108). Therefore, the differential functional and metabolic properties of prostate epithelial cells in the peripheral zone may determine their high potential for malignant transformation. It has been reported that the dorsolateral lobe of the rat prostate is embryologically homologous to the peripheral zone of the human prostate (109). The dorsolateral lobe also retains higher levels of zinc compared to the ventral lobe (110). However, the mechanisms for the uneven distribution of zinc within the prostate are unknown. Previous study done by Hirano et al detected higher expression of ZnT2 in the dorsal and lateral lobes of rat prostates than in ventral lobes by using semiquantitative RT-PCR (110). The current study quantitatively assessed that ZnT2 transporter expression was about 1000 times higher in the dorsolateral lobes than in the ventral lobes where ZnT2 mRNA levels were very low. These findings suggest that ZnT2 may play a role in accumulating high concentration of zinc in prostate dorsolateral lobe. However, more studies are needed to address the mechanism and significance of this lobe-specific expression of ZnT2.

In summary, the current study suggests that ZnT2 may play an important role in regulating zinc homeostasis and maintaining high zinc levels in the prostate dorsolateral lobes. The current study provides evidence and directions for future research to explore the possible interactions among prostate cancer, zinc status, and defects in zinc transporter families.

Table 5.1 Primers of genes analyzed by qRT-PCR.

Gene name	Primers	Sequence (5' → 3')	Annealing temperature	Amplicon size (bp)
Zip1	Forward	AAGCCTAGTGAGCTGCTTCG	58	148
	Reverse	ATGGCCAGGATGAACTCTTG		
Zip2	Forward	TTCAGAAATTCGTGGTGCAG	58	140
	Reverse	GCGACTCCAAAAGGAAGACA		
Zip3	Forward	CGTCTTCCTGGCTACATGCT	58	148
	Reverse	TCCACGAACACAGTGAGGAA		
Zip4	Forward	ATGAGCTGCCTCACGAACTT	58	130
	Reverse	CTGCTAGAGCCACGTAGAGG		
ZnT1	Forward	CACGCTAGTGGCTAACACCA	60	296
	Reverse	AGGAAAACACGGGTTCACAC		
ZnT2	Forward	TGCTCGTGTACCTGGCTGTA	60	138
	Reverse	TCCATGTCCAGACTGATGGA		
ZnT3	Forward	GCAGAGTATGCACCACTGGA	60	202
	Reverse	CAAGGGCGCAGATAGAGAAG		
ZnT4	Forward	CCTTTGGATTTCATCGCCTA	60	144
	Reverse	GTTCTCTGCACAGCCTCGTA		
18S	Forward	GGACCAGAGCGAAAGCATTTGC	60	115
	Reverse	CGCCAGTCGGCATCGTTTATG		

Table 5.2 Zinc concentrations in the plasma and tissues of the rats fed the MZD, MZD +P or ZA diet[1]

	MZD	MZD+Phytase	ZA	P-value
Plasma (µg/ml)	0.56±0.03[a]	0.90±0.06[b]	1.15±0.05[c]	<0.0001
Prostate				
Ventral lobe (µg/g)	11.92±0.80[a]	14.39±0.91[a]	12.49±0.70[a]	0.09
Dorsolateral lobe (µg/g)	57.54±1.19[a]	186.60±1.25[b,c]	199.53±1.12[c]	0.0009

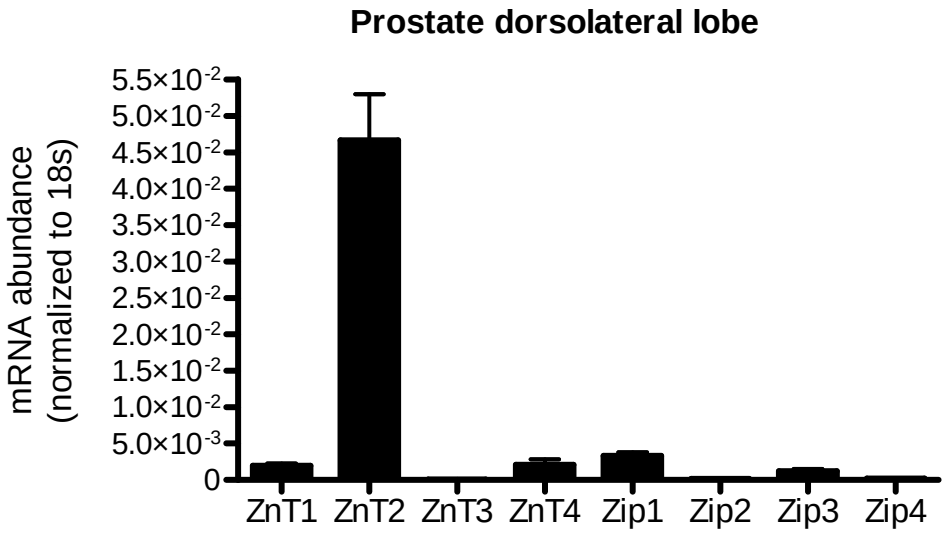
[1] Values are means ± SEM (n=12). Main effects were determined by one-way ANOVA with Bonferroni's post-hoc test. Means without a common letter differ, $P<0.05$.

Table 5.3 Correlations between zinc transporter mRNA levels and zinc concentrations in the dorsolateral lobes[1]

	Correlation coefficient	*P*-value
ZnT1	0.04	0.81
ZnT2	0.38*	0.034
ZnT3	-0.20	0.28
ZnT4	-0.18	0.32
Zip1	0.24	0.18
Zip2	0.35	0.053
Zip3	-0.12	0.50
Zip4	-0.14	0.45

[1]Pearson analysis was performed to analyze the correlation, n=32. *, $P < 0.05$.

A



B

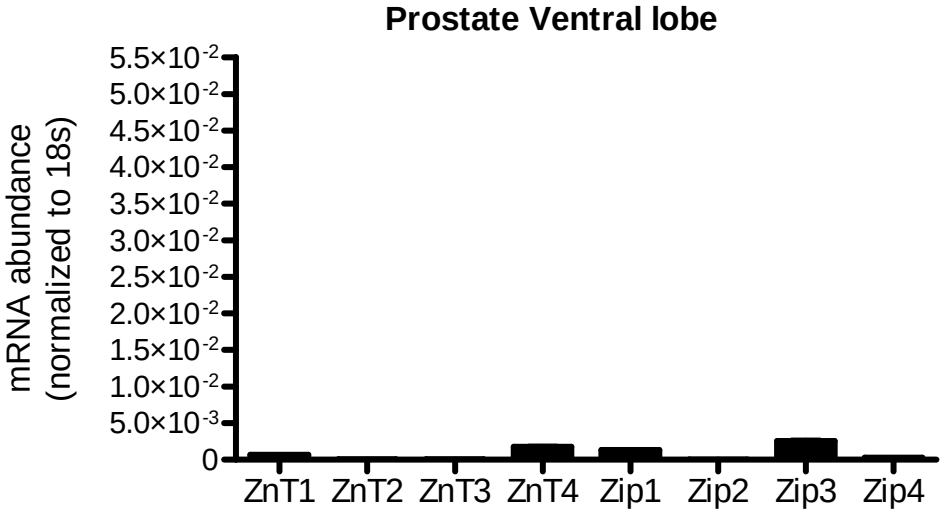


Figure 5.1 Zinc transporter mRNA abundance profiles in the (A) prostate dorsolateral and (B) ventral lobes. Sprague-Dawley rats were fed a ZA diet for 9 weeks. mRNA levels were measured as described in *Material and Methods*. Results are transcripts copy numbera normalized to 18s transcripts. Values are means ± SEM (n=12).

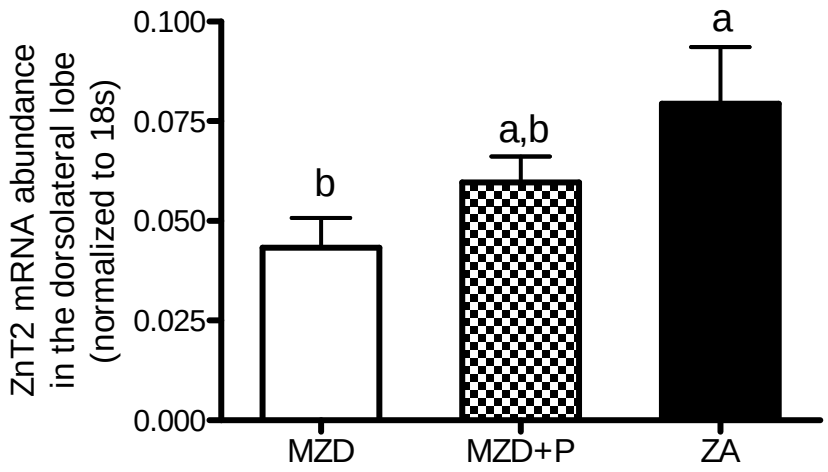


Figure 5.2 Effects of dietary marginal zinc depletion on ZnT2 mRNA abundance in the prostate dorsolateral lobes. Sprague-Dawley rats were fed a MZD, ZA or MZD+P diet for 9 weeks. mRNA levels were measured by qRT-PCR. Results are transcripts copy numbers normalized to 18s transcripts. Values are means ± SEM (n=12). Difference between means is determined by one-way ANOVA followed by Bonferroni's post-hoc test. Means without a common letter differs (P<0.05).

Chapter 6 General conclusions

Diet is estimated to account for one-third of preventable cancer, and dietary micronutrient deficiencies may increase the risk of cancer by causing DNA damage. Extensive research has been done to explore the effects of folate, B6 and B12 on DNA integrity, and the role of zinc in maintaining DNA integrity has been recently recognized (142). Approximately 12% Americans do not consume the Estimated Average Requirement (EAR) of zinc, thus a significant proportion of U.S population could be at risk for marginal zinc deficiency (147). Zinc plays a significant role in cell proliferation and apoptosis, defense against free radicals and DNA damage repair. Previous studies done in our lab have shown that zinc depletion in cells increased DNA damage *in vitro*. However, the *in vivo* effects of zinc deficiency on DNA integrity are still unclear and the mechanisms for it are still unknown and understudied. Thus one focus of this dissertation was to explore the effects of zinc depletion on DNA integrity *in vivo* zinc, and to understand the mechanisms of these effects.

Our studies confirm that dietary zinc depletion impairs DNA integrity *in vivo*. This dissertation shows that both severe and marginal zinc deficiencies are capable of increasing DNA strand breaks in peripheral blood cells of rats, but this DNA damage is reversible and could be reduced to baseline levels by zinc repletion. We also observed similar effects in human subjects with marginal zinc depletion and repletion, indicating that zinc is essential for maintaining DNA integrity in humans.

At least two mechanisms are likely involved with the effects of zinc deficiency on increasing DNA damage. First of all, zinc deficiency compromises the antioxidant effects of zinc and increases oxidative stress. The increased oxidative stress or increased susceptibility to exogenous oxidative stresses causes oxidative DNA damage directly. Secondly, zinc deficiency affects DNA repair functions and interferes with the activities and expressions of DNA repair proteins, including p53 and PARP. p53 is a zinc-containing protein and plays an essential role in regulating DNA repair, cell proliferation and cell death (65). Poly ADP ribose polymerase (PARP) is also a zinc containing protein and plays an essential role in normal cellular functions, such as DNA replication, cell responses to DNA damage and DNA repair (171). Since zinc deficiency interferes with the responses of p53 and PARP to DNA damage, the DNA

repair functions of the cells are impaired. Altogether, during zinc deficiency there is a dual effect where both the generation of DNA damage is increased and the cell's ability to repair DNA damage is lost, resulting in a substantial accumulation of DNA damage.

Zinc concentration in the prostate is the highest of any soft tissue and is proposed to be essential for prostate health. This dissertation shows that marginal zinc deficiency sensitizes the prostate to exogenous oxidative stress, chronic exercise. Marginal zinc deficiency or chronic exercise alone had no effects on DNA integrity in the prostate; however, a combination of both marginal zinc deficiency and chronic exercise markedly increased oxidative DNA damage in the prostate. We showed that marginal zinc depletion only decreases zinc concentrations and affects DNA integrity in the dorsolateral not ventral lobes. This data suggests that the prostate dorsolateral lobe, the area of the prostate most susceptible to cancer, was more sensitive to marginal zinc deficiency than the ventral lobe. Our studies for the first time demonstrate that the prostate is highly susceptible to DNA damage with low dietary zinc intake. Although high zinc levels in the prostate could be essential for prostate health, the mechanisms by which the prostate maintains zinc homeostasis are still unknown. This dissertation suggests that ZnT2 in the prostate may play a major role in accumulating high zinc levels in the prostate dorsolateral lobe and regulating prostate zinc homeostasis during marginal zinc depletion. Future studies will examine the molecular mechanism by which ZnT2 maintain zinc homeostasis, and examine the regulation of zinc transporters during disease conditions, such as aging and cancer.

The current biomarkers for zinc deficiency, such as plasma zinc and hair zinc, have poor sensitivity and specificity (178), which hinders the identification of susceptible populations. Thus, the identification of early, sensitive biomarkers of zinc status is a critical issue in the field. This dissertation shows that DNA integrity in peripheral blood cells is more sensitive to dietary changes in zinc than the plasma zinc. Therefore, measuring DNA strand breaks in peripheral blood cells could be used as a complementary assay to traditional biomarkers to more sensitively identify human zinc deficiency.

In conclusion, this dissertation supports the essential role of zinc in maintaining DNA integrity *in vivo*. It also emphasizes that zinc is important for the prostate's ability to defend itself against exogenous oxidative stress, and to maintain DNA integrity. Moreover, this dissertation contributes to the knowledge of the understanding of the mechanisms of maintaining prostate zinc homeostasis, and identified ZnT2 as the main functional zinc transporter in the prostate. Overall, the current study highlights the importance of adequate-zinc nutriture for DNA integrity and prostate health, and provides the basis for future study in examining the connection between zinc and DNA damage related diseases.

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