EVALUATION OF NEW TECHNIQUES FOR ZINC AND CORTISOL ASSESSMENT WITH A PLACEBO-CONTROLLED ZINC SUPPLEMENTATION TRIAL IN A SUBSAMPLE OF ETHIOPIAN WOMEN

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Submitted to the Faculty of the
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in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 2011

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ABBREVIATIONS

AIDS = Acquired immune deficiency syndrome
AGP = α-1-acid glycoprotein

BMI = Body mass index

cAMP = Cyclic adenosine monophosphate
Cd = Cadmium
CDF = Cation diffusion facilitator
CO_2 = Carbon dioxide
CRP = C-reactive protein
Cu = Copper
CuZn-SOD = Copper zinc superoxide dismutase
CYS = Cysteine
Cys_2His_2 = Cysteine histidine

EAR = Estimated average requirement
EDTA = Ethylene diamine tetraacetic acid
EIA = Enzyme based immuno assay
ELISA = Enzyme linked immunosorbent assay

FAO = Food and Agricultural Organization
FDA = Food and Drug Administration
FRAP = Ferric reducing antioxidant power/ferric reducing ability of plasma

GR = Glucocorticoid receptor
GRE = Glucocorticoid response element

HCO_3 = Bicarbonate
HIS = Histidine
HIV = Human immunodeficiency virus
HRP = Horseradish peroxidase

ICP-MS = Inductively coupled plasma mass spectrometry
IgG = Immunoglobulin G
IGF-1 = Insulin-like-growth factor-1
IL-6 = Interleukin-6
IP = Inositol phosphate
IRMA = Immunoradiometric assay
IZiNCG = International zinc nutrition consultative group

K = Potassium
kDa = kilodalton
KLF4 = Krüppel-like factor 4

Li = Lithium

mRNA = Messenger ribonucleic acid
MT = Metallothionein
MTF-1 = Metal response-element binding transcription factor

Na = Sodium

OSU = Oklahoma State University

PBMC = Peripheral blood mononuclear cells
PCR = Polymerase chain reaction

RDA = Recommended dietary allowance
RIA = Radioimmuno assay
RNI = Recommended nutrient intake
ROS = Reactive oxygen species
RT-PCR = Real-Time/Reverse Transcriptase Polymerase Chain Reaction

SD = Standard deviation
SEM = Standard error of the mean
SLC = Solute carrier family
SNNPR = Southern Nations Nationalities and Peoples Region

T_3 = Triiodothyronine
T_4 = Thyroxine
TPEN = N,N,N′,N′tetrakis(2-pyridylmethyl)-ethylenediamine
TSH = Thyroid-stimulating hormone
TTP = Tristetraprolin

UL = Upper level
UV = Ultra-violet light

WHO = World Health Organization

CHAPTER I

INTRODUCTION

The 2005 Ethiopian Health and Demographic Survey found that women are the key to determining a population policy. They are the main target group in attempts to decrease fertility and to increase female participation in all spheres of education. The Ethiopian government actively worked towards the provision of women with all social, economic, and legal rights, despite traditional practices. Data showed that the empowerment of women improved the health standards of both the women and their families (1). As a continuation of this argument, analysis and assessment of health status of women in developing countries require increased attention. Current research attempts not only to focus on the identification of important variables related to nutritional status, but also to focus on socioeconomic indicators such as household size, income distribution and education (2). While several studies have been performed in the Sidama region of Ethiopia, none of these studies were concerned with non-pregnant women (3-5).

In order to gain an in-depth view of the health status of rural populations in developing countries, additional parameters besides iron, iodine, and protein have to be assessed. A more profound knowledge of the health status of women would enable governments and healthcare providers to appropriately address problems. One of the health indicators gaining increased attention in developing countries is zinc status. Lack

of zinc in the diet has a severe impact on immunity and growth. It causes a type II deficiency as defined by Golden (1996), which implies that nonspecific symptoms, mainly growth retardation and immunological changes, are detectable long before the body zinc is completely diminished (6, 7). As a result of this zinc sparing mechanism, zinc in accessible tissues does not necessarily reflect nutritional status (8). Zinc deficiency is widespread and severe in developing countries and varies from mild to moderate within the developed nations of the world (9). Low meat consumption paired with a high phytate and/or fiber intake contributes to deficiency in developing countries. Affected the most are young children, pregnant and lactating women, and the elderly. To alleviate these deficiencies, international research activities are pursuing supplementation and fortification policies of the targeted groups, along with improved methods of dietary diversification (9, 10).

Zinc has been identified as important in human nutrition since 1964, but adequately assessing zinc status remains a challenge (11, 12). Confounding results in the literature originate from the lack of reliable methods of assessment (13). Therefore, to learn more about the zinc status, a reliable method for large scale assessment of zinc status is required. Zinc measured in plasma or serum may not necessarily reflect tissue concentrations (8). Total dietary zinc intake does not take into consideration the bioavailability (14), and height-for-age as a measurement is not very specific even in children, because of confounding factors (13, 15, 16). Cell culture studies in intestinal cells show that there is a causal link between low zinc intake and high mRNA expression for zinc transporter SLC39A4, Zip4 (17, 18). Thus, the dietary dependency of Zip4 expression makes this transporter a potential biomarker for zinc intake. Furthermore,

Zip4 mRNA expression is significantly correlated to zinc availability in Caco-2 cells, a human mucosal cell line (19).

The development of a non-invasive method, for example in buccal cells, to determine zinc status would potentially enhance the knowledge about zinc and increase nutrition understanding in the field, not only in the developing world but also globally (20). Based on the fact that low dietary zinc is associated with oxidative damage of DNA, measuring the amount of DNA damage has the potential to provide important information about the zinc status of an individual. The single cell electrophoresis method, also called the comet assay, can be used to evaluate damage and repair mechanisms of cells, the latter by monitoring the cells over a certain amount of time after induced damage (21). This method measures the degree of damage to nucleated cells by oxidative stress and has been successfully used both in cell models (22) and in human samples (23).

Zinc status is also associated with cortisol (24). Cortisol is a glucocorticoid hormone from the hypothalamic-pituary-adrenal (HPA) axis that acts as an important stress marker. Stress of any kind, such as anxiety, strenuous exercise, inflammation or malnutrition triggers the excretion of cortisol from the adrenal glands (25, 26). For instance, protein energy malnutriton is often seen in conjunction with low zinc intake and increased serum cortisol concentrations. Elevated cortisol in turn suppresses inflammatory cytokines and prevents an overwhelming inflammatory reaction (27-29) by downregulating the expression of inflammatory cytokines (30, 31). In addition, cortisol induces the expression of mRNA and protein for the zinc finger protein tristetraprolin (TTP), which suppresses inflammatory cytokine expression. In lung endothelial cells for example, TTP bound to the 3’ region of TNF-α mRNA and

suppressed its expression post-transcriptionally. Cortisol levels are increased by physiological stress of any kind (32). The development of a tumor, for example, raises cortisol corresponding to the increased wasting process. The adipose tissue contains zinc-α-glycoprotein, a lipolytic factor which corresponds well with the cortisol levels of cachetic patients (33). Current literature on cortisol indicates that the assessment of inflammatory markers when combined with cortisol is an effective tool for evaluating cell damage (34). The possibility of using saliva as a specimen simplified the assessment of cortisol significantly. A comparison of the results between saliva and the established methods for cortisol analysis reveals that the results are often significantly correlated (35, 36).

Purpose

The purpose of this study was to evaluate new possibilities for assessment of changes in zinc intake. Therefore, changes in zinc transporter Zip 4 (SLC39A4) expression in buccal cells were assessed with polymerase chain reaction (PCR). In addition, the plasma zinc concentrations of 40 Ethiopian women at the beginning and end of a placebo-controlled zinc supplementation trial were measured, as were several other health indicators. One of the health indicators, cortisol, was measured in saliva, serum, and urine in order to assess the correlation between the three measurements. To assess the antioxidative effects of zinc supplements in blood, the comet assay method was employed.

Objective

1. To assess plasma zinc and other health indicators in serum (albumin, ferritin) and urine (iodine, total protein), as well as acute phase proteins (C-reactive protein, α-1-acid glycoprotein) before and after a double-blind, placebo-controlled zinc supplementation trial in Southern Ethiopia.
2. To assess changes in the mRNA expression of the Zip 4 zinc transporter in buccal cells due to supplementation with zinc sulfate for 17 days, and to relate the expression of Zip 4 (SLC39A4) mRNA to the measured plasma zinc concentration.
3. To compare the cortisol levels measured in saliva, urine and serum.
4. To study the change in antioxidant capacity due to zinc supplementation with the ferric reducing antioxidant power (FRAP) method, and to study the change in oxidative stress with the comet assay.

Null hypotheses

$H0_1$: There will be no significant difference between plasma zinc of the supplemented and placebo groups at baseline and after 17 days of treatment with 20 mg zinc taken as daily tablets.

$H0_2$: There will be no significant difference in the health indicators (serum ferritin, albumin, urinary iodine, and total protein) or acute phase proteins (hsC-reactive protein, α-1-acid glycoprotein) in either group at baseline and endpoint of the trial.

$H0_3$: There will be no significant change in the expression of Zip 4 transporter (SLC39A4) mRNA and metallothionein mRNA due to zinc supplementation.

$H0_4$: There will be no correlation between cortisol measured in saliva, serum and urine samples. In addition, there will be no significant change in cortisol comparing baseline to endpoint in both groups.

$H0_5$: There will be no significant antioxidative effect due to the zinc supplement, as measured with either the FRAP or the comet assay.

Assumptions and limitations

It is assumed that no contamination of samples in the field occurred, and that the time required for transport from the field to the laboratory as well as to the laboratories at Oklahoma State University and Oregon State University did not damage the samples.

A possible limitation of this study is that it was performed in January, shortly after several important religious festivals. Therefore, one to two months prior to the study the volunteers were probably eating slightly larger amounts of food, especially meat than they were during the rest of the year. Furthermore, January is in the harvest season, which further increases the food intake. Due to these changes in food availablity, the measured biochemical parameters might not be representative of other seasons (37).

One difficulty in interpreting the data of this study is the lack of studies in non-pregnant women with the same type of diet as our study group. There is a very limited pool of studies performed in Ethiopia, and most of the scientific interest is focused on

pregnant women, children, and groups with severe health impairments, such as HIV/AIDS or tuberculosis. Comparing our data to the findings of those studies was somewhat difficult, because hemodilution in pregnancy and growth in children increase zinc needs, while comorbidities change some health parameters. In addition, livelihood, agricultural production, and diet are relatively distinct in different areas of Ethiopia, making comparison with data from other regions difficult (1, 38, 39).

Furthermore, we did not assess whether the women were lactating. Lactation might increase their need for certain nutrients. In additon, pregnant women were excluded based on the women's own report, not based on pregnancy test results.

Organization of the dissertation

The introduction is followed by a review of literature, and a materials and methods section. The results section was divided into several single research papers. A short conclusion is provided at the end of the dissertation, followed by suggestions for future research.

CHAPTER II

REVIEW OF LITERATURE

Study area and population

Ethiopia has a population of roughly 90 million in a geographic region about twice the size of Texas. It is one of the poorest countries in the world, with a Gross Domestic Product per capita of US$ 900 (ranking 213 out of 227 counties). Approximately 85% of the Ethiopian workforce is employed in the agriculture sector (40). Land is mainly owned by the government and only loaned to the farmer, which makes it difficult for the farmer to make middle and long-term investments (41). Middle and long-term investments would be necessary to make a rain-fed agriculture less vulnerable to drought (42). Research also shows that increasing population pressure reduces the amount of cultivated land per farmer, and yet farmers are working together less than they used to in previous years. The isolation of farmers eventually leads to more land fragmentation and poverty (41). Sharing assets and workforce in agriculture would not only increase food security but decrease the potential for conflicts (43).

Around 3.5% of Ethiopians belong to the Sidama ethnic group (40). Geographically the Sidama region reaches from the Great East Africa Rift Valley lowlands (1500 meters above sea level), with the lakes Awassa and Abaya, to the Arbegona highlands (3000 meters above sea level) in the eastern districts of Arbegona,

Bansa, and Arroressa. The regional capital of Awassa is 275 km south of Addis Ababa, the national capital (Figure 1) (44). The Sidama region has been part of Ethiopia since 1891, when it was acquired through a takeover by King Menelik II. Several attempts to gain more autonomy within Ethiopia have ended in bloodshed. The last such event was in 2002, when 71 Sidaman civilians allegedly died in the outskirts of Awassa (45).

Figure 1: Map of study region. Sudan and Eritrea are to the north, Kenya to the south, and Somalia to the south-east of Ethiopia. The red circle indicates the Awassa region, in which the village of Finichawa lies.

Sidama has its own customs and a strict behavior code. In the past, private and community landownership paired with economic cooperation made Sidama one of the wealthiest regions in this part of Africa, but this well-established system was destroyed in 1891 (44). Agricultural production in the Sidama region is based on relatively reliable rainfall, therefore food insecurity is a more recent phenomena based on the increasing population. Even during the famine of 1984, which affected all of Ethiopia, Sidama subsidiary agriculture kept its food production stable. However, the farmers have little

access to markets and only an inadequate credit system is available to them, leading to low levels of production and high dependency on traders. The main crops produced are coffee, khat, corn, and enset. Corn and enset are the food staples of this region, while coffee and khat are the main sources of income for the farming families (46, 47). There is little manufacturing industry in the whole of Sidama, except a few government-owned and some privately owned factories. Unemployment and underemployment in this densely populated area are therefore at an estimated 70% (46).

In conclusion, poverty and food insecurity in the study region are based on population pressure and structural problems in agricultural production. Furthermore, a lack of opportunities to work outside of subsistence farming and to gain access to markets also contributes to impoverishment of villagers of the Sidama region.

Study population

International research ought to promote the most desirable ways of improving living standards, nutrition, education and health, and women today are seen as the key to food security (48). Therefore, this study targeted women of a farming community. The women who volunteered for this study lived in the village of Finichawa, which has approximately 5000 inhabitants and lies at about 2000 meters above sea level, 15-20 kilometers from Awassa. Data from Ejana-Wolene, another enset planting region in the south of Ethiopia, showed strong gender differences in household chores; while most men engage in farming, trading, and other manual work, women engage in the household chores and food selling. Considering the time spent for all chores, women work much longer hours than men and earn significantly less than their spouses (49). Significant

female contributions to family farming and animal husbandry suggest that the role of Ethiopian women in this agrarian society is tied to agricultural production and that researchers and administrators have given too little attention to women (50). However, data looking at the production of field crops showed that an older child contributes more overall days of field work than an adult woman (51).

An important question when recruiting volunteers for studies in Africa is the prevalence of HIV/AIDS. Data collected in 1994 from Addis Ababa showed that HIV prevalence was 6.9% in adult women, with a peak at 25-29 years of age (11.8%) (52). The 2005 HIV/AIDS rate for the Awassa region in Ethiopia was around 9.5%, down from 14.4% in 1989, according to a WHO report (39). Research from Tanzania shows that HIV prevalence is much higher in trading centers than in rural villages with less mobility, fewer bars and less risky sexual behavior. Prevalence was generally higher in women than men (53). Due to the fact that Finichawa is primarily a farming community, the HIV/AIDS prevalence is probably low.

In conclusion, this study deliberately targeted non-pregnant women, as most of the studies in the area have been conducted in pregnant women and children. Women in rural areas work long hours and have minimal access to resources. Based on our estimations, the HIV/AIDS prevalence in the targeted village is most likely well below 10%.

Nutritional status and diet of the study population

The Ethiopian Hunger Index, based on data from different sources, showed that in the year 2000, hunger was most prevalent in The Southern Nations Nationalities and

Peoples Region (SNNPR) of all regions in Ethiopia. SNNPR had the highest calorie deficits for families and malnutrition in children. However, the numbers for SNNPR improved the most of all Ethiopian regions from 2000 to 2005. Rural areas are more food insecure compared to urban settlements in rural areas. In addition, inhabitants of rural areas have less access to adequate sanitation and healthcare (37).

The body mass index (BMI) is one indicator for food insecurity. BMI patterns in adult women follow availability of food, with a maximal BMI in the post-harvest period and a minimum BMI in the pre-harvest time (51). A sample from an enset growing society (collected from January to March) showed that both men and women had low BMI (<18.5) in about 30% of the cases. The younger women and older men had slightly higher BMIs compared to their age matched control group (49). This low BMI indicates an impaired long term health status. In nutritional studies and in food security studies, more attention should be given to adults' food patterns and weight changes, which are better indicators for seasonal changes in food availability than weight patterns of children, because children may experience greater changes in weight due to diarrhea episodes than due to food insecurity (51).

The diet in rural areas of the Sidama region is mostly plant-based, with cereals, mainly unrefined maize (*Zea mays L.*) as unleavened bread (58.7%), and enset (*Enset ventricosum*) (29.7%) being the main energy sources. The combination of maize and enset in one meal is common in rural areas in Southern Ethiopia (51). A higher amount of enset compared to maize intake results in lower protein, fat, iron, zinc, fiber and phytate in the diet, while calcium is significantly increased due to a higher calcium content in enset compared to maize. While a study in the Sidama region revealed higher amounts of

B_{12} in the diet and plasma of volunteers with increased enset consumption, due to fermentation of enset, researchers also found some early functional deficiencies of B_{12} measured as methylmalonic acid, total homocysteine, and cystathionine (4). Sidamans consume only small amounts of animal-source foods, such as cow's or goat's milk with coffee. Meat is only eaten during religious festivals. Livestock products are used primarily for income-generation (3).

Consistent with the plant-based diet, research studying the dietary intake in pregnant volunteers from Sidama showed that zinc, protein and calcium intakes were very low. The researchers found that nearly 100% of the volunteers were below recommendations for protein intake and plasma zinc. Less than 10% had iron deficiency anemia, but 74% were at risk of low calcium intake based on diet records (3).

A further health problem is iodine deficiency, typical for a landlocked country. Worldwide patterns are that close to the coastal areas, evaporated seawater (50 µg/L) saturates the soil with iodine. In these areas iodine deficiency is no problem. But washed out soils, far away from the coast, such as those in mountain regions, do not contain sufficient iodine, and plant concentrations can be as low as 10 µg/kg dry weight (54).

In the study region goiter rate in adults was found to be 76.6% where cassava is a stable and 54.9% in non-cassava eating regions. Within the non-cassava eating regions, the number of iodine-deficient habitants was significantly increased in higher altitudes, such as in Sidama, than in lower altitudes, probably due to increased washout of soils in the mountains compared to the low lands. For instance, urinary iodine in higher altitudes was significantly lower (mean 58.0 µg/L) than in low lands (mean 75.4 µg/L) (55). In girls 6-12 y of the SNNPR, 39.1% had palpable goiters and 17.3% had visible goiters

(56). Women with goiters had a significantly higher chance of reproductive failure, such as miscarriage or stillbirth, than women with no goiter (16.7% vs. 13.8%). Due to the stigma, girls with visible goiters are often not sent to school and are not wanted for marriage. Researchers found that the knowledge base concerning IDD is minimal. Over 95% of the women in the region did not know about the importance of iodized salt and its connection to goiter, and traditional practices like washing salt further reduce the iodine content (55). Only 2.2% of the households in SNNPR were using iodized salt in 2005 (56).

An important aspect of the Ethiopian diet is the fermentation of food. Fermentation is a low-cost food preservation and preparation technique, which improves food safety and quality. The inoculation often happens by chance with different genii of lactic acid bacteria and yeasts (57). The production of B vitamins, in fermentation, mainly B_{12} and folic acid, depends mainly on the bacteria and yeasts used. While some bacterial strains produce B_{12} and folic acids, others are capable of destroying it (58). The volunteers in the studied villages might be protected against B_{12} deficiency due to the intake of fermented and baked enset pulp as their main staple food (4). Fermentation not only increases B-vitamins, but also more importantly decreases the phytate content of food by reducing the large inositol phosphate groups of phytate to lower inositol phosphate groups (59). Phytate, the main storage form of phosphorus in plants, has higher inositol phosphate groups and impairs the uptake of zinc and iron from the diet. Fermentation increases the bioavailability of zinc and iron by reducing the phytate content. For example, while the phytate to iron molar ratio in unfermented fresh kocho

pulp from enset is 0.9:1, and the phytate to zinc molar ratio is 8.4:1, the fermented product has non-detectable amounts of phytate. Unleavened cornbread, however, has a phytate to iron ratio of 5.4:1 and phytate to zinc ratio of 21.6:1, the latter clearly limiting bioavailability (60). Another study calculated an overall diet phytate to zinc ratio of 17:1, which could contribute to low plasma zinc (5). A study from Nigeria, however, showed that enzymatic dephytinization with a wheat phytase rather than fermentation of maize porridge increased the zinc uptake in children (5 to 10 y), with a fractional uptake of dietary zinc of 55.5% in phytase treated samples versus 30.5% in fermented samples (61). However, low molecular weight acids produced by bacteria may increase the uptake of zinc and iron by forming soluble ligands. Mild acids increase the phytase activity of bacteria, which further decreases the chelating properties of phytate (59). The consumption of foods high in ascorbic acid, such as kale and tomato, may increase the effect of the fermentation further (60).

The geographic region should be taken into consideration when examining plant composition. Comparisons of analyzed data from unleavened maize bread in different parts of Ethiopia showed large discrepancies in the phytate and iron, but not zinc content. Bread (100 g) from the Gamo region contained 459.6 mg of phosphorus, 10.7 mg of iron and 2.6 mg of zinc, while the same amount of bread from the Tigray region contained only 174.1 mg of phosphorus, 2.7 mg of iron and 1.2 mg of zinc. Bread from the Sidama region had 217.2 mg phosphorus, 3.2 mg iron and 1.2 mg zinc. Baked enset, the other staple of Sidama, also had a different composition depending on the region. In Sidama, 100 g baked enset contained 69.2 mg of phosphorus, 3.1 mg iron, 0.6 mg of zinc and 208 mg of calcium; in the Gedeo region, however, it contained 30.7 mg of phosphorus, 11 mg

iron, 0.6 mg of zinc and 76 mg of calcium (62). Iron and zinc deficiencies are usually strongly connected (63), but this is not the case for the Sidama region. One study showed that even in pregnant women with higher needs of iron, the dietary iron intake was mostly sufficient, only 32.3 % had low iron stores, and only 8.7 % had iron deficiency anemia (3).

An important contributor to low bioavailability of nutrients in general is fiber, a part of which is phytate. A plant-based diet high in unrefined grains and legumes contained high amounts of fiber (64). Based on diet analysis data from this region, the most concentrated sources of fiber intake were legumes. Chickpeas (5.5 g fiber in 100 g of boiled product), lentils (2.6 g), and kidney beans (2.9 g) contain fiber well above the amounts found in any of the staples in the Sidama region (62). However, legumes were eaten in much smaller amounts than maize, therefore contribute less fiber to the entire diet (3).

In general, the calcium content of the non-dairy food items eaten in the region was relatively high. Cooked legumes had an especially high content of phytate and calcium, however, the literature shows that calcium may not further impair the bioavailability of zinc and iron (65, 66). High calcium content was found in fermented kocho (162 mg/100 g), boiled kidney beans (60 mg/100 g) and teff (124-155 mg/100 g), while barley (45mg/100 g) and maize (12-16 mg/100 g) were lower (60). The calcium content of plant-based foods is in general much lower than in the best sources, milk and dairy (67). Although some plants are relatively high in calcium, their bioavailablity is low, possibly contributing to low serum calcium concentrations found in pregnant women (3).

In conclusion, diets in the Sidama region are monotonous and may not provide for the nutritional needs of a farming community. Fermentation of some food products does improve the bioavailablility slightly, but high phytate content in the unleavened maize bread and legumes decreases the uptake of trace minerals. Furthermore, the high intake of goitrogenic substances, in conjunction with limited access to iodine-fortified food items, makes iodine deficiency a large problem in the area.

Zinc

Physiological function of zinc

Zinc is an essential micronutrient but can be toxic for the cells; therefore, its uptake and distribution in the body is closely regulated (68). Zinc is very important for many functions of the body, including growth, immunity, and as an antioxidant. Up to 10% of the human genome encodes for proteins with zinc-binding domains (69). Zinc homeostasis in humans is regulated on two levels, uptake from the intestine and excretion through intestinal secretions, such as bile (70).

Zinc is a major contributor to growth in children. For example, the intake of highly bioavailable zinc by pregnant women is significantly correlated to the birth weight of their children (71). Impaired growth in pre-pubertal children can, at least in part, be corrected by long-term zinc supplementation. Linear growth is seen to be dependent on sufficient zinc intake at this critical age of accelerated growth (72).

An overview article by Powell (73), a pioneer in the field, shows that zinc exhibits antioxidant properties. However, it does not fit the historic definition of an antioxidant as it does not directly quench free radicals, molecules with one or more unpaired electrons.

Instead, researchers distinguish the chronic and acute antioxidant properties of zinc (73). The chronic antioxidant properties operate through metallothionein (MT), a zinc-containing, cysteine-rich protein, which has antioxidant properties. MT is, for example, active in many tissues in order to detoxify ethanol or to protect from the effects of radiation to name only a few of its many effects. Chronic deficiency of zinc decreases MT and therefore makes the body more susceptible to oxidative damage due to redox reactions. MT is releasing Zn^{2+} in order to keep the redox state of the cell constant, however the exact mechanism is not yet known (73, 74). Research suggests that glutathione disulfide might play a role in the transfer of zinc to the particular site of action (75). Chronic antioxidant properties of zinc are based on its redox potential, whereby it protects certain proteins from oxidation by binding to them (e.g., to sulfhydryl groups). Zinc has also antagonizing pro-oxidation reactions through iron and copper. Free iron and copper will bind to protein structures, DNA, or membrane proteins, where they initiate site specific oxidative injury, such as lipid peroxidation, by forming free radicals within these structures. Zinc is able to antagonize the redox potential of these metals, so they are not able to attack protein structures; zinc is even thought to enter protein structures and replace iron and copper (73).

Zinc can also be considered an acute antioxidant, mainly through enzyme activity, such as copper zinc superoxide dismutase (CuZn-SOD), which is found in the cytosol as well as in the plasma, while manganese superoxide dismutase can be found in mitochondria. Based on its abundance, CuZn-SOD is seen as one of the important antioxidant systems in the human body (76). In preeclampsia patients, for example, serum zinc and erythrocyte CuZn-SOD concentration and activity were significantly

decreased compared to a matched sample of pregnant women with no signs of preeclampsia. The data suggest that the development of preeclampsia is associated with minimal zinc intake and therefore low levels of CuZn-SOD (77).

The effect of zinc on cell integrity is crucial. Zinc is an intracellular trace element of which large amounts can be found in the nucleus. There it has an important function in transcription, as an antioxidant and in repair processes (78, 79). Cell models show that zinc deficiency leads to increased oxidative DNA damage and reduces the ability of transcription factors containing zinc finger structures to bind to DNA sequences. Therefore, although DNA repair mechanisms were initiated, due to the fact that the binding to DNA was reduced, the proper repair of the damaged DNA was impaired (80) (22). CuZn-SOD is a key to antioxidant protection of cells, suggesting that zinc has a significant role in the prevention of cancer (81).

A zinc deficiency study in a rat model supported the notion of increased oxidative damage to the DNA, while repair mechanisms, for example through protein p53 DNA binding, were impaired (74). The cancer suppressor p53 is upregulated due to zinc impairment in the cell, but the binding ability of p53 is reduced due to the zinc deficiency, which in turn reduces the structural integrity of zinc finger proteins (22). Song et al. (2009) found it difficult to work even with pair-fed groups in a rat study, as the zinc deficiency decreased the food intake by 46%. The pair-fed group therefore did not receive adequate amounts of zinc, indicated by 20% lower hepatic zinc concentrations than the zinc adequate group (74).

Zinc depletion through dietary restriction (1 week with 0.6 mg/d and 5 weeks with 4 mg/d) in nine men led to increased oxidation, measured as DNA breaks, while the

repletion (1 week with 20 mg/d and 4 weeks with 11 mg/d) reversed the effect. Plasma zinc levels during depletion did not significantly change and stayed around 79 µg/dL. In the repletion period, the plasma zinc levels increased significantly to 86 µg/dL ($p < 0.05$) compared to the measurement at the end of the depletion period. The common oxidative marker, total antioxidant capacity (FRAP), did not change ($p = 0.37$) over the course of the zinc restriction and repletion. Arachidonic acids and F_2 isoprostane in plasma as well as erythrocyte SOD activity did not change significantly during the depletion and repletion period. However, DNA-breaks tended ($p = 0.09$) to decrease and F_2 isoprostane ($p = 0.001$) decreased significantly between the two baseline measurements (day 1 and 13 with zinc intake of 11 mg/day), indicating a marginal zinc deficiency prior to the study, with slightly increased DNA breaks on day 1 compared to day 13 (23). Additional data obtained from the same group of men during the above mentioned study supports the idea that acute zinc intake is more important than long term intakes, as the fractional absorption in the intestine made up for fluctuations in intake. Researchers showed how fast the body's fractional zinc absorption reacted to changes in zinc intake during the two-step zinc depletion (1 week plus 5 weeks) and repletion (1 week and 4 weeks) period. During deficient and sufficient intake, the plasma and urine zinc levels were largely unchanged (82).

Excessive production of nitric oxide increases Zn^{2+} ions in the cell due to a disruption of the binding of zinc to protein structures, among other processes (83). Free Zn^{2+} ions in turn could possibly damage the cell and create oxidative stress. Some of the excess zinc can be stored by MT which reacts to increases of dietary zinc with a higher expression of MT mRNA (68).

In summary, zinc is crucial for many functions in the body, and has an important role as an antioxidant, acutely as CuZn-SOD and more long-term as MT zinc. It is involved in several mechanisms that support the integrity of the cell and DNA. Fractional absorption accounts for fluctuations in dietary intake, while plasma and urine zinc concentrations may not change.

Endogenous and exogenous sources of zinc

There are two main sources of zinc: intrinsic due to digestive secretions, and extrinsic, through diet. The endogenous sources of zinc are pancreatic and bile secretions. These are rich in zinc metalloproteins, which release zinc as soon as they are hydrolyzed in the lumen. Dietary sources of zinc are grains and animal products. Some cereals contain appreciable amounts of zinc, with whole grain cereals containing more zinc than refined cereals (84). Still, the best dietary source of zinc is meat. Zinc bioavailability from animal sources is much higher than from plant sources, due to the fact that uptake is supported through protein (71). From meals containing beef with radiolabeled zinc, around 20% of the zinc was absorbed by humans (85). The best source for zinc is red meat; other good sources are liver, fish, shellfish, nuts and legumes. Poor sources of zinc are vegetables and fruits (11).

In the Ethiopian diet, barley is a good source of zinc, while other staples such as maize, teff, enset, legumes and vegetables contain amounts well below 1 mg of zinc per 100 mg product (wet weight). The zinc content of local tilapia and catfish has not yet been assessed. Data on local fish of the Reo area reveals that the zinc content is around 2

mg per 100 mg dried and boiled product, well above any dairy source, but below meat. For example, fried goat from the Afar region contains 6.4 mg of zinc in 100 mg (62).

In conclusion, the diet of farming villages in the Sidama region is unlikely to provide for sufficient amounts of bioavailable zinc. Plants and local fish most likely do not make up for the lack of meat, the most bioavailable source of zinc.

Dietary factors contributing to zinc deficiency

Zinc deficiency is widespread in developing countries and can still be found in developed countries. The main reason for zinc deficiency is poor dietary intakes (86). Although zinc content is somewhat high in bran and germ, these grain components are also high in phytate, a phosphorous storage molecule, which impairs the uptake of zinc (9, 84). A diet with a phytate to zinc ratio of 23 for only 9 days significantly reduced plasma zinc concentrations in young and elderly women (87). However, not all phosphorus-containing components of plants form chelates with zinc and minerals in foods. Factors responsible for lowering bioavailability are mainly hexa-inositol phosphate-6 (hexa-IP-6) and penta-inositol phosphate-5 (penta-IP-5), which belong to the higher inositol phosphates (88, 89).

In developing countries where women and children are especially likely to have low meat intake, diets are also typically high in fiber and phytate. This may also be true for certain diets found in developed countries (e.g., the vegan diet). Low meat consumption and high intake of minimally processed grains are suspected to be the root cause of impaired zinc status. In plant-based diets with small amounts of animal-source foods, multiple nutrient deficiencies are likely to develop (64, 90).

Several researchers explored the possibility of dephytinization of food with acid in an effort to increase zinc bioavailabilty. This method reduced the phytate to zinc ratio in cereals from 11 to 0 and had a significant effect on the bioavailability shown in smaller amounts of stable zinc isotopes excreted in feces. However plasma zinc did not increase using crossover tests in adults with two days of treatment with stable isotopes and 2 to3 weeks washout period (91). Additionally, a high calcium intake is thought to contribute to the malabsorption of zinc by enhancing the effect of phytate and results in indigestible calcium-zinc-phytate complexes (11, 84, 92, 93). A high fiber diet also contributes to impaired zinc uptake by decreasing zinc bioavailability (84). Furthermore, a radioisotope study in rats shows that high intake of polyphenols commonly found in coffee, such as caffeic acid and chlorogenic acid (an ester of the caffeic acid), impaired the uptake of zinc by 25% but did not have an effect on copper uptake (94).

A further reason for zinc deficiency might be geophagia, which is practiced throughout the world. Depending on the regional soil mineral composition, geophagia has a distinct effect on health. In some cases it can be seen as a natural multi-mineral supplementation. In Africa geophagia is often utilized during adolescence and pregnancy. Clay may contain high amounts of calcium, but also lead and cadmium. Geophagia reduces morning sickness in women by reducing the motility of the stomach. However, research suggests that clay is responsible for the slow release of calcium and other minerals, thereby reducing bioavailability (95).

In conclusion, human diets can be categorized into diets with a high, moderate, and low bioavailability for zinc. The high bioavailability diet has around 50% available zinc and is based on highly refined cereals and adequate proteins from animal sources,

and therefore has a phytate to zinc molar ratio of < 5. The moderate bioavailablility diet has around 30% of the zinc available and a phytate to zinc molar ratio of 5-15. It consists of a mixed diet with an animal protein source. However, if the intake of unrefined cereals exceeds 50% of the caloric intake and the cereals are not fermented or germinated, or if geophagia provides a high intake of inorganic calcium salts (> 1g Ca^{2+} ions), the molar ratio is above 10. The low availability diet has less than 15% zinc bioavailability, with phytate to zinc molar ratios of well above 15 (96). It is high in unrefined, ungerminated and unfermented cereals with no significant source of animal protein.

Requirements for zinc

The body reserve of zinc is very small, at only about 2 g or 30 mmol in adults; of this, 60% is in muscles and 30% in bones. The highest concentrations of zinc can be found in the choroid of the eye (4.2 µmol/g or 274 µg/g) and the prostatic fluids (4.6-7.7 mmol/L or 300-500 mg/L) (97).

Because there is no ideal and sensitive indicator for zinc status, recommendations for zinc intakes are estimations based on studies found in the literature. Furthermore, several studies showed a certain adaptation process to low zinc intakes, a mechanism which has to be taken into account when estimating requirements (98). The daily recommendation for adult women was set at either 36 µg/kg body weight (assuming a bioavailability of 50% dietary zinc) 59 µg/kg body weight (bioavailability of 30% dietary zinc), or 119 µg/kg body weight (bioavailability of 15% dietary zinc). The WHO recommended nutrient intakes (RNI) are 3 to 9.8 mg zinc per day (high to low bioavailability, normative amounts) and an upper limit of 35 mg per day for women with

55 kg body weight to meet the normative storage requirements (99). The IZiNCG Estimated Average Requirement (EAR), based on the Institute of Medicine (IOM) EAR **(**providing sufficient intake for 50% of the population) is 7 mg per day for unrefined plant-based diets and 6 mg per day for refined plant based diet for women weighting 55 kg (100, 101). The Recommended Dietary Allowance (RDA), covering 98% of the U.S. population needs for zinc, suggests 8 mg dietary zinc per day for women 19 years and older (102).

During pregnancy the need for zinc increases and goes from 4.4 to 8.0 mg zinc per day (high bioavailable zinc source) to 14.7 to 26.7 mg/day (low bioavailable zinc source) from the first to third trimester. Based on the output during the first 3 months, lactating women theoretically have three times the need of non-lactating women; however, the postnatal involution of the uterus and skeletal storage makes up for the additional need. The zinc recommendation goes from 7.6-5.8 mg/day (high bioavailability) to 25.3-19.2 mg per day (low bioavailability) for the lactation period of 0 to12 months (99). The EAR for women 14 to 18 years old recommended by IZiNCG is 9 mg per day from a refined plant-based diet or 12 mg per day from an unrefined plant-based diet during pregnancy. The recommendations during lactation for the respective diets are 8 or 9 mg per day. For women above 19 years old, the IZiNCG recommendations are slightly lower, 8 or 10 mg per day during pregnancy and 7 or 8 mg per day during lactation (100, 102). The RDAs for pregnant or lactating U.S. women are 12 or 13 mg for the age group 14 to 18 years of age, and 11 or 12 mg for 19 years and above (102).

Large variations in absorptive efficiency of zinc were found in growing children and teenagers, and also in adults where it ranges from 20-50% (103). With an assumed range of 25% variation in uptake, the recommended nutrient intake (RNI) for adult women (19-65 y, with assumed body weight of 55 kg) was set at 3 mg per day in high bioavailability diets to 9.8 mg/day in low bioavailability diets (99).

The upper level (UL) for U.S. adults 19 years and older is 40 mg per day; in pregnant and lactating women of all ages, the UL is 34 mg per day, based on data suggesting CuZn SOD impairment above these levels (102). These concentrations are only reachable with excessive amounts of supplements, certain types of seafood, a contaminated water source, or galvanized cooking utensils. Although zinc toxicity is seldom seen, acute intoxication with 4 to 8 g leads to vomiting, nausea, fever, lethargy and diarrhea. The long-term intake of high zinc can lead to impairment in the absorption of copper, and to a certain extent, iron. Impairment of copper has to be taken into consideration when supplementing with zinc (102, 104).

In conclusion, zinc has the highest bioavailability from meat sources. The plant components phytate and fiber impair zinc uptake from the gut and increase the fecal excretion of zinc from endogenous secretions. Acute or chronic toxicity of zinc are seldom seen.

Impact of zinc deficiency and its indicators

As there is little body reserve of zinc, low intake rapidly leads to mild zinc deficiency with levels below 700 µg/L. Severe deficiency occurs mainly in developing countries, in areas with a plant-based diet high in phytate, and/or through malabsorption

due to intestinal parasites, diarrhea, and impaired utilization through genetic diseases (e.g. sickle-cell anemia, acrodermatitis enteropathica). All of these factors lead to zinc deficiency in infants, children, and adolescents. During pregnancy, zinc deficiency may occur due to elevated requirements (105). Zinc deficiency is also responsible for loss of appetite, hypogeusia (blunting of sense of taste), dermatitis, night blindness, and alopecia (hair loss). Furthermore, poor growth, stunting, skeletal abnormalities, and inadequate cartilage and collagen are typically due to reduced cell division. In addition, zinc deficiency disrupts insulin-like-growth-factor-I (IGF-I) synthesis, which mediates the cellular effects of growth hormone. However, there are other factors that reduce IGF-I, so a supplementation of only zinc does not necessarily increase growth (106-108). A zinc depeletion of previously sufficient young men over seven weeks did not show reduced neutrophil zinc or reduced activity of neutrophil α-D mannosidase and alkaline phospatase, despite a significant reduction in plasma zinc. Erythrocytes, having a slow cell turnover, did not show any significant changes in zinc content of whole cells or membrane during the depletion and repletion. Likewise, platelet count and platelet zinc concentration did not change (109).

Because zinc has so many different functions, zinc deficiency affects numerous pathways. The metalloenzymes are severely affected as zinc binds amino acid residues and enables structural integrity in all major enzyme groups. Zinc plays an important role in gene expression, cell replication, membrane and cytoskeletal stabilization, and hormone structure (11, 110). In addition to impaired cellular growth, zinc deficiency leads to impaired immune function. Zinc is an important part of the three levels of the physical defense system, including cell-mediated immunity antibody production, and

phagocytosis. Furthermore, unbound zinc has a direct antiviral effect on rhinovirus replication (111). Zinc is essentially needed by highly proliferative cells and has several important additional functions. For example, zinc is imperative to skin and mucosal cell health (112-114). An example of zinc deficiency dermatitis is the often misdiagnosed dermatitis in newborns. Premature infants are mainly affected but term infants can also suffer from severe skin lesions, due to the low zinc content of breastmilk paired with their increased needs (115).

Figure 2, based on Fraker and King (2004), shows the effect of zinc deficiency on the immune system and cancer in a simplified form. Thereby the effect on cortisol levels was also included.

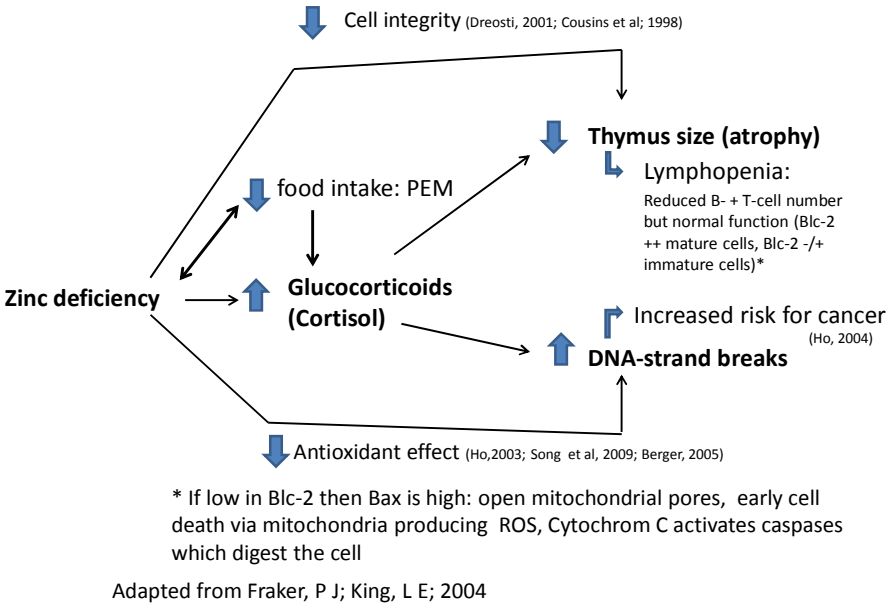

Figure 2: Effects of zinc deficiency and cortisol increase on cell integrity, thymus size, DNA strand breaks and antioxidative capacity, adapted from Fraker & King, 2004 (30).

The reduction of the zinc-dependent hormone thymulin, involved in T-cell proliferation and maturation, leads to atrophy in the thymus, which is the site of T-lymphocyte maturation. This contributes to poor immune function and wound healing (1). During infection, the plasma zinc decreases and hepatic concentration rises. More MT is up-regulated in liver cells and is further induced by cortisol and IL-6, to reduce the circulating zinc available for bacterial use (117).

In addition to its immunological effect, zinc deficiency may also influence the thyroid function. Previous research examined the interrelationship between zinc and thyroid hormones, but the data are inconclusive (118). In an animal study, both zinc deficiency and hypothyroidism through low iodine intake are connected to decreased food intake and therefore weight loss. For example in rats with hypothyroidism, hyperthyroidism, and zinc deficiency (< 1mg Zn/kg diet compared to 30 mg Zn/kg diet), weight loss was found to be significant compared to pair-fed and the controls of each group (119). In addition, no interaction effect for the weight loss between the groups was found. The rats' tissue weight loss depended mainly on thyroid function and the reduced food intake, rather than the zinc deficiency. However, zinc deficiency had an interaction effect on both hyperthyroid and hypothyroid ($p < 0.05$) in respect to insulin-like growth factor 1 (IGF-1). The deficiency significantly reduced serum IGF-1 in hypothyroid and hyperthyroid rats (compared to pair-fed groups, $p < 0.001$). In addition to IGF-1, zinc status influenced growth via zinc-containing growth hormone transcription factors and response elements rather than through T_3 protein directly (119, 120). In humans however, hypothyroidism is clearly associated with weight gain, lethargy, cold intolerance, and hoarseness of the voice (121).

Supplementation with zinc should be considered in certain diseases. For example, zinc supplementation decreases the severity and course of diarrhea in children and should therefore be added to the treatment where deficiency is prevalent (122). A pooled analysis of several studies with children below 5 years of age showed that therapeutic zinc supplementation did decrease the duration and severity of acute (by 15%) and chronic (by 25%) diarrhea. Only in approximately half of the studies did plasma zinc increase significantly due to supplementation (123).

Zinc deficiency has a negative effect on the body through suppression of enzyme function due to the lack of available zinc for proper structure and functioning. One suggested contributor to zinc imbalance is defects in the proteins that are important for the homeostasis of zinc, like the zinc transporters or MT (124).

In conclusion, due to the fact that zinc plays an important role in many functions of the body, zinc deficiency has no single effect, but impairs processes on several levels. One of the most important effects of chronic zinc deficiency is the impairment of the immune system, which can cause severe problems. Furthermore, zinc deficiency might influence the thyroid function.

Zinc deficiency in the study population

Few zinc-related research studies have been conducted on non-pregnant women in Ethiopia (84, 125). Nevertheless, most of the studies conducted on children and pregnant women have indicated severe deficiencies with negative impacts on health (126, 127, 128). From a study among pregnant women of the Sidama region, the results indicated severe shortness (height for age 18 z-scores of <-2SD) in 30% of the volunteers.

Furthermore, approximately 72% of the women had inadequate dietary zinc intakes based on the IOM EAR (3, 4, 102, 129, 130). In the Gondar region of Ethiopia serum zinc of pregnant HIV-positive and negative women was within the normal range (131). However, the researchers did not mention the material they used for the blood collection, which could be a source of contamination. In another sample collected in the same region, a quarter of the control subjects had zinc deficiency based on serum concentrations (132).

Most information about zinc intakes and plasma zinc in the region is for pregnant women. The findings indicate that dietary intakes of protein, energy, iodine, zinc, calcium, and to a certain extent iron are low in rural villages of Sidama.

Supplementation with zinc and possible outcomes

Long term therapeutic supplementation with zinc is often used in diabetics, as they are prone to be zinc deficient (133). Zinc supplementation can have a positive effect on antioxidant status in this group (134). Studies showed that even a small deficiency in zinc may lead to oxidation of LDL and VLDL even in healthy adults (81, 135). High amounts of oxidized cholesterol and lipoproteins increase the activity of macrophages and the buildup of atherosclerotic plaques (136).

Furthermore, zinc supplementation can be used to assess the effect of changes in zinc intake on other health parameters, such as growth. In children of developing countries, zinc supplementation reduces incidents of diarrhea and pneumonia (137). Supplementation trials, followed by plasma zinc and height-for-age data, are seen as some of the best ways to measure zinc status in a population (138).

Supplementation with 15 or 30 mg zinc as zinc gluconate daily for 6 months in men aged 58 to 68 years of age, showed significant dose dependent increases in plasma zinc. The supplementation had no significant effect on urinary and erythrocyte zinc (139). Zinc supplements below the upper level have no known long term side effects which might be expected, such as impaired copper, iron or manganese bioavailability. For such an impairment to exist, a long term exposure or very high doses would be required (140).

In two studies by the same group, short term zinc supplementation with 50 mg zinc per day as zinc gluconate in healthy men showed a significant increase in plasma zinc after only 6 to7 days. The plasma concentrations decreased somewhat but stayed significantly above the baseline and above the placebo group after 10 to 15 days of supplementation (141, 142). In a documented but unpublished study by Morejohn and Brown, 26 healthy U.S. men received a supplement of 15 mg per day and showed significant increases in serum zinc concentrations compared to baseline data. The pattern shown in the two studies by Sullivan et al. (1997 and 1998), with a significant increase at 6 to 7 days followed by a slight decline toward baseline at 10 to15 days, was not observed by Morejohn and Brown (143).

In conclusion, although zinc supplementation is in general a good method to assess the zinc status on a population level, supplementation trials have their limitations. The increase of plasma zinc is not linear due to supplementation. Furthermore, it is difficult to determine if zinc supplementation produces a significant effect on zinc in different compartments in the body.

Zinc digestion, absorption and metabolism

The presence of chelators, inhibitors, or enhancers, and food preparation processes such as heat treatment influence bioavailability of metals (87). For example a low pH increases digestion and probably the absorption of zinc into the enterocytes. While the exact mechanism of enhancement is not yet known, antacids like H_2-blockers reduce zinc uptake (11, 144).

Although zinc uptake occurs throughout the length of the small intestine, zinc is mostly absorbed in the jejunum (145). Like any other mineral, zinc absorption depends on size and degree of polarity; small non-polar minerals diffuse rapidly through the lipid bi-layer on the apical side of the enterocytes. The carriers most active in zinc transport in the intestine are Zip4 (Zrt/Irt-like protein 4) and ZnT5 (zinc transporter 5). The expression of both transporters is increased in the case of zinc deficiency and decreased with adequate zinc (70). However, it is not yet clear if zinc is bound to a ligand in a chelated complex, or if it is transported as a free ion through the brush border (11, 87). Zinc transport through Zip4 does not depend on ATP, K^+ or Na^+, but might be facilitated through Zn^{2+} HCO_3^- co-transport (146). Zinc uptake from a high meat diet was 28 to 30% (total of 3.6 mg/d), the same percentage as from an otherwise identical low meat/zinc-containing but otherwise identical diet (2.0 mg/d). The uptake from a third diet with low meat content but enriched with supplements (zinc gluconate) was around 18% (2.1 mg/d). However, the excretion through urine was significantly higher from the high meat diet (0.33 mg/d) compared to both other diets (0.26 mg/d) (147). Excretion through the feces was similar in the high meat diet and the supplemented group (10.9 and 10.7 mg/d), while the low meat group lost only 6.5 mg/d. Overall the high meat group had a significantly

increased balance of +1.8 mg/d. The low meat group experienced a net loss of -0.02 mg/d, and the low meat supplemented group gained 0.66 mg/d (147).

Zinc absorption into the portal circulation has two main mechanisms, transcellular and paracellular transport. The transcellular transport is carrier-mediated, while the paracellular transport occurs through simple diffusion. When zinc concentrations in the lumen exceed the carrier capacity, simple diffusion dominates and increases the uptake of zinc into the lamina propia (87). Basolateral zinc is then bound to albumin in the plasma and transported to the liver. There 60% of the zinc is bound to albumin, and 15 to 40% is bound to α_2-macroglobulin, immunoglobulin G (IgG), and transferrin. Finally 2 to 8% is bound to two low molecular-weight amino acids, histidine and cysteine (148).

Due to the fact that 60% of the zinc in serum is bound to albumin, inflammation causes a significant drop in plasma zinc along with albumin. Likewise, a significant loss of muscle tissue due to protein energy malnutition increases zinc levels in the blood (149).

Based on the information given in previous sections, zinc absorption varies substantially and is strongly dependent on the diet. Diets with generally low zinc intakes, such as vegan or vegetarian nourishments, have an increased absorption compared to diets high in meat, even though it is not clear how the homeostatic regulation works (87, 150).

In conclusion, the most effective way to increase zinc status is by eating meat. Zinc supplements are also somewhat effective, but not to the extent of meat. Although zinc homeostasis, uptake through the gut, and excretion in urine and feces can

compensate for some dietary fluctuations, these adapted mechanisms can only partly make up for constantly low intakes.

Transport of zinc within the cells

Cousins and coworkers have described their findings concerning zinc transport in many studies (70, 151, 152). Before the first transporter was identified in 1995, zinc transport was thought to occur through co-transport mechanisms only. Trans-membrane zinc transporters may belong to two gene families, the ZnT (SLC30A) (zinc transporter) (solute carrier family) and the Zip (SLC39A) (Zrt/Irt-like protein transporter). The ZnT transporters of the SLC30A family are also called cation diffusion facilitators (CDF) because they transport zinc out of the cells or into specialized organelles within the cell such as mitochondria, endoplasmic reticulum, Golgi apparatus, endosomes, or lysosomes (153). ZnT reduces the intracellular zinc concentration by releasing zinc out of the cell or sequestering it inside vesicles. The uptake of zinc into all eukaryotic cells and the release from vesicles are managed by the solute carrier family 39A (SLC39A) of which 14 members are identified, belonging to one of four subfamilies. Members of the subfamily II are Zip1, 2, 3, while the Zip4 belongs to subfamily LIV-1 (70, 151, 152). Zip transporters increase the cytosolic zinc availability by increasing the uptake into the cells and releasing zinc from vesicles. After endocytosis, zinc is transported through the endosomal membrane to enter the cytosol, where it binds to MT (70).

In conclusion, there are two important zinc transporter families, and both contribute to the homeostasis of zinc in the body. The different zinc transporters work

together. For example, in the enterocytes, several transporters are responsible for zinc uptake out of the lumen and excretion into the blood.

Transcription factors involved in zinc homeostasis

The major transcription factor involved in zinc homeostasis in mammals is considered to be metal-response-element binding transcription factor 1, also referred to as metal responsive transcription factor 1 (MTF-1), which was first identified in 1988. MTF-1 binds upstream with promoters of genes which are crucial to metal homeostasis for inducing transcription of genes. The up-regulation of the transcription of genes of the ZnT family and metallothionein (MT) through an increased availability of zinc is a known process. In essence, increased dietary zinc leads to an increased phosphorylation of MTF-1 and increased activation of metal-responsive element (MRE), expressing more MT and ZnT in enterocytes, both of which reduce the amount of zinc available to the cell (154, 155).

Zinc transporter ZnT and MT transcription are regulated through various cell signaling pathways activating MTF-1. Dietary or environmental metals trigger the cascade that leads to increased phosphorylation of MTF-1 and finally to an upregulation of ZnT and MT mRNA. Various kinases perform the phosphorylation; among them are casein kinase II, tyrosine kinase, and other protein kinases. This highly phosphorylated MTF-1 is then accumulated in the nucleus, binds to MRE of the gene, and starts ZnT or MT transcription. Inhibitors of these kinases did not inhibit phosphorylated MTF-1 from binding with MRE, but researchers hypothesize that incompletely phosphorylated MTF-1 protein residues undergo changes that suppress transcription instead of binding with

MRE. However, cAMP-dependent protein kinase II, involved in glycogen, glucose, and lipid metabolism, does not appear to play any role in zinc induced transcription (155, 156). A cell-culture study showed that for MTF-1 to bind to MRE, high concentrations of zinc inside the cell are needed (154).

MTF-1 contains six zinc finger elements, of which not all bind with the same affinity to MRE and zinc (Figure 3). While N-terminal zinc finger elements one to four will bind with high affinity to MRE and zinc, the two remaining C-terminal elements do not bind very tightly. However, eliminating zinc fingers five and six would destabilize the MTF-1 and lead to a loss of binding capacity to MRE (157).

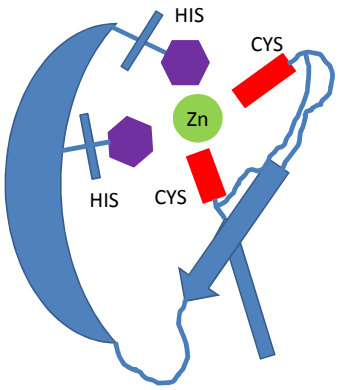

Figure 3: Single zinc finger motif with two histidine (HIS) and two cysteine (CYS) proteins holding the zinc ion.

The above mentioned dissimilar binding capacity of the six Cys_2His_2 motifs gives MTF-1 a certain zinc sensing property. However, because zinc binds to other components of the cell as well, zinc binding ability in vivo is lower than the potential calculated binding capacity. Binding also depends on the metal; for cadmium the measured affinity

was several times larger than for zinc. Within the six single zinc finger motifs (position 1-6), the affinity for zinc varies from 1.1 to a factor of 20, with the motif on position 4 binding the strongest then position 2 and 5, both bind stronger than the motif in position 6 which is binding stronger than 3 and 1 ($4 > 2 \approx 5 > 6 > 3 \approx 1$). This indicates that the central zinc finger motifs (2, 4, 5) have a higher affinity than the peripheral ones (1, 6). The low zinc affinity of the zinc finger motifs 6 and 1 makes them the most likely candidates as intracellular zinc sensing factors. However, the zinc sensing character of these zinc finger motifs is not strong enough to react to free zinc ion concentrations within the cell (158, 159).

Zinc transporters ZnT and MT are transcripted via phosphorylated MTF-1 binding to MRE which can be found in several sites of the genome. Increased dietary zinc has been shown to induce the expression of ZnT and MT mRNA. Research is not conclusive as to whether zinc finger motifs have zinc sensing abilities.

Krüppel-like factor 4

The down-regulation of the MT and ZnT and up-regulation of Zip transcription during zinc deficiency are less well understood (160). Zip4 is transcribed via the Krüppel-like transcription factor 4 (KLF4) rather than MTF-1 (161). Like many transcription factors, KLF4 consists of three highly conserved zinc finger motifs, each consisting of two cysteine and two histidine molecules (Cys_2His_2) (162). During zinc deficiency, large amounts of transcription factor KLF4 and Zip4 could be found in mouse epithelial cells. Furthermore, the destruction of KLF4 caused a significant decrease of Zip4 in a murine intestinal epithelial cell model (161). KLF4 is expressed in endothelial

cells, monocytes, B-cells and corneal epithelia (163, 164). Prior to transcription, KLF4 goes through several steps of activation. The transformation also can lead to a degradation of KLF4, and the cell cycle goes on (165). If KLF4 is not degraded, the up-regulated KLF4 leads to growth arrest, for example by suppressing genes that promote biosynthesis of proteins and cholesterol (163, 166). However, KLF4 has a very ambiguous role in cells. It is on one hand a cancer promoter and on the other a cancer suppressor, depending on the action of p21, which might switch the tumor suppressor p53 on or off, depending on other signals (167). DNA damage, for example, triggers the expression of p53, which activates KLF4. This process can go in different directions; if p21 is also activated, KLF4 can lead to cell cycle arrest, and at the same time suppress apoptosis (Figure 4) (168).

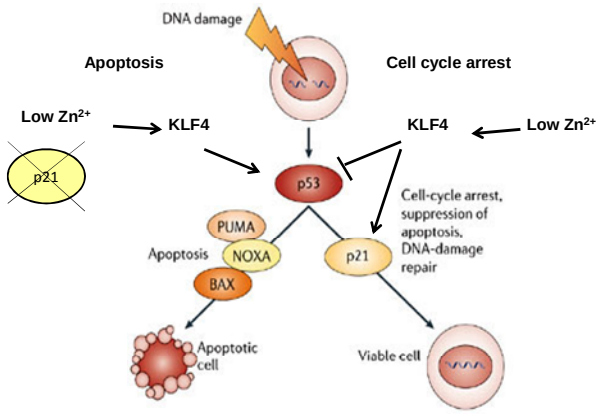

Figure 4: KLF4 pathway leading to apoptosis or cell cycle arrest due to low zinc availability. Two pathways are possible, apoptosis or cell cycle arrest. In the case of apoptosis, p21 will not be activated, while in the case of cell cycle arrest, p21 can suppress the apoptosis and start the repair of the viable cell. In apoptosis BAX plays an important role in the apoptosis of the cell (adapted from Rowland & Peeper, 2006) (167).

Zip4 mRNA stability apparently is decreased due to increased zinc uptake, and therefore the regulation of Zip4 is thought to be mainly post-transcriptional and post translational rather than pre-transcriptional. For example, the transcription of Zip4 was not down-regulated with adequate zinc intake, but the post transcriptional expression and the localization of Zip transporters changed. With increased dietary zinc intakes, Zip4 mRNA was not stable, and therefore less Zip4 protein was found in cells. Furthermore, the Zip4 proteins were localized inside the cell in zinc adequate diets, rather than in the membranes (169). There must also be an inhibitory effect of MTF-1 towards KLF4. MTF-1 might be suppressing the attachment of KLF4 to the MRE binding site (170).

In conclusion, when not enough zinc is available to the cell, Zip4 will be up-regulated via the KLF4. Due to the fact that the activation of KLF4 triggers growth arrest or apoptosis in cells, zinc deficiency may lead to decreased growth.

Specific role of Zip4

Zip4 transporters were found in human cells in the stomach, proximal small intestine, distal small intestine, pancreas, liver, and the visceral yolk sac. These transporters are not found in skin, muscle, heart, kidney, brain, spleen, ovaries, testes or seminal vesicles (151, 171, 172). The molecular weight of Zip4 is 70 kDa, determined with anti-SLC39A4 antibodies in Caco-2 cells, a model for epithelial cells. The comparable weight for Zip4 from cDNA of the same cells is 72 kDa and a smaller peptide at 35 kDa. These numbers suggest that the proteins are in a non-glycosylated form in the intestinal cells (171, 173).

Several investigators found a close connection between zinc deficiency and Zip4 mRNA expression. For example, a study using Caco-2 cells, as a model for human intestinal cells, showed a clear down-regulation of Zip4 mRNA measured by RT-PCR in zinc concentrations of 3 μM, 100 μM and 200 μM in the media for 14 days (173). Animal models demonstrated similar results. For example, zinc deficiency induced the transcription of Zip4 mRNA in the small intestine of pregnant mice after only 24 hours of deficiency. In visceral yolk sac, the increase of Zip4 mRNA expression started on day 5 of the deficient diet, while Zip5 and Zip1 expression did not change. Furthermore, in adequate zinc status, immuno histochemical assessment with rabbit antiserum against human Zip4 showed intracellular Zip4 protein in mice intestine samples instead of Zip4 proteins at the cell surface. In zinc deficiency in mice, Zip4 protein was found in the apical part of the enterocytes in order to increase uptake. In β-cells of the pancreas, insulin and Zip4 protein can also be found, however, the concentration of Zip4 stayed unchanged during deficiency (171).

While many studies have been conducted using rodent models, double-blind placebo-controlled studies of humans suggest a down-regulation of Zip4 mRNA expression in zinc supplemented groups. One study used intestinal cells of the volunteers, gained through pinch biopsies of illeal mucosa after 14 days of supplementation with 25 mg of zinc (173). Another study measured changes in Zip3 mRNA expression, instead of Zip4, to assess dietary changes. The supplementation with 15 mg zinc for 16 days in young men found a significant down-regulation of Zip3 and an up-regulation of MT mRNA in leucocytes and whole blood from dried blood spots of the supplemented group (174). Although the above mentioned studies indicated a down-regulation of Zip4 mRNA

during zinc supplementation, this could be due to a post transcriptional degradation of the mRNA, rather than a diminished transcription, as suggested by researchers (169, 175).

The zinc transporter Zip5 was found localized with Zip4 in many tissues (171). Tissue samples indicate that Zip5 might be responsible for the excretion of zinc through the small intestine and pancreas. In enterocytes Zip5 is located on the basolateral membrane, where it might be removing zinc from the blood; however, in zinc deficiency Zip5 is localized to intracellular vesicles (171).

To conclude, the uptake of zinc increased in zinc deficiency, thereby the Zip4 mRNA was upregulated and Zip4 protein levels also increased. Supplementation with zinc decreased zinc uptakes, through decreasing Zip4 mRNA levels. Research indicates that the mRNA expression in both cases stayed initially the same; however, in the case of adequate zinc, mRNA seems to be post transcriptionally degraded in a dose-dependent manner.

Other zinc transporters

In the past few years, researchers have used new technologies to identify additional zinc transporters, and their biochemical role(s) for the body. They tried to identify the most important transporters in body compartments such as blood and tissues (171, 172).

Among cells, the most interesting for zinc research in human blood are monocytes, leucocytes and erythrocytes. Human monocytic leukemic cell-lines (THP-1) and peripheral blood mononuclear cells (PBMC) obtained from supplemented volunteers, for example, serve as models to study the effect of dietary zinc fluctuations in immune

cells. The use of the zinc chelator N,N,N',N'-tetrakis (2 pyridylmethyl) ethylenediamine (TPEN) (< 5 μM) increased the Zip2 mRNA expression significantly in these models (176). Another study in THP-1 cell cultures by the same group found that zinc depletion through TPEN chelation caused decreased mRNA expression of Zip1 and MT, but increased Zip2 mRNA expression. In addition, the most rapid change was seen in ZnT1 mRNA expression (180). In another test, in which THP-1 cells were stressed through dietary zinc fluctuation, researchers found that although Zip1 mRNA and ZnT5 mRNA were the most abundant genes, ZnT1 and Zip2, and to a certain extent ZnT7 mRNA were the most sensitive towards changing zinc homeostasis. During zinc depletion, ZnT1 and ZnT7 mRNA expression decreased significantly, while Zip2 mRNA expression increased. With high dietary intakes of zinc, MT and ZnT1 mRNA levels increased significantly, while Zip2 decreased (18).

Although zinc transporters are important as possible markers of dietary zinc, most also transport other metals (177). For example, Zip2 appeared to be an ideal target as an indicator of changes in zinc availability. Tests in erythro-leukemia K562 cells showed that Zip2 also transported metals such as copper, magnesium, iron, and cobalt. Zip2 is found on the extracellular surface of the plasma membrane of K562 cells. The inhibitory effect with >6 fold excess of the metals was compared to 3 μM zinc in K562 cells. Fe^{2+} and Co^{2+} had no influence on the endogenous zinc uptake while Cd^{2+}, Cu^{2+} and Mg^{2+} significantly inhibited the Zn uptake into the K562 system. These findings suggest that Zip2 is transporting other metals in addition to zinc. In addition, the uptake of zinc by Zip2 was increased when HCO_3^- was present, suggesting a cotransport role of HCO_3 (146). Furthermore, a study found that Zip2 is not essential under normal conditions, but

gains more importance during pregnancy. The study in Zip2 knockout mice showed that zinc, calcium and iron homeostasis were negatively affected during pregnancy, but not in the non-pregnancy stage (178).

Zip14 might also have a role in zinc homeostasis. During inflammation, zinc in the plasma was diminished. Researchers found that an up-regulation of transporter Zip14 mRNA expression by pro inflammatory factors led to an increased zinc and iron uptake in mouse liver cell models, suggesting a possible mechanism for diminished plasma zinc during inflammation (17).

In summary, not much is yet known about each transporter and their role in zinc homeostasis. Although some zinc transporters react to dietary fluctuations, the co-transport of other metals makes it difficult to use them as indicators for dietary changes of zinc intake. Apart from Zip4, the transporters ZnT1, Zip2 and Zip14 are the most promising candidates for measurements of dietary zinc intake.

Storage and excretion of zinc

There is no significant storage of zinc in humans. Zinc content in the human body is mainly intracellular and totals between 1.5 to 2.5 g in adults. With high zinc intake, increased amounts of zinc can be localized in the bones, but it cannot be mobilized from the bone during deficiency except during the normal turnover of bones (11, 92).

Metallothionein (MT), a cysteine rich binding protein, may provide a pool of intracellular zinc. Currently, ten MT genes have been identified in humans (155). Humans barely react with MT expression to increased levels of mercury. (179). High zinc and cadmium intakes, however, increase the production of MT (156). A study in Caco-2

cells showed increased levels of MT mRNA expression during supplementation, in accordance with the given concentration (173). In the case of high levels of available zinc, the metal will bind increasingly to MT, exercising MT's detoxifying function (11, 70). MT is found in the cytosol and in mitochondrial membrane. It is produced when high amounts of zinc, copper, or toxic heavy metals are found in the cells. Higher levels of MT lead to a down-regulation of the uptake of more zinc into the cells of enterocytes, the liver, and the kidneys (152). Studies in humans show that during zinc supplementation, MT mRNA transcription, measured with qPCR methods, increased significantly in leukocytes extracted from dried blood spots (174, 180). Dietary changes are visible not only through changes in the expression of mRNA, but also through changes in the protein, assessed with a sandwich ELISA technique using anti-human MT antibodies. Supplementation with 80 μM zinc increased the MT mRNA and protein levels significantly in THP-1 cells, while zinc depletion of THP-1 cells with TPEN (5-10 μM) decreased MT expression levels (180). Tests in peripheral blood mononuclear cells (PBMC: 85% lymphocytes and 15% monocytes) from supplemented (15 mg/d zinc) or placebo-treated patients show that MT mRNA was more sensitive than protein towards dietary increases of zinc and showed depletion prior to detectable apoptosis (176).

A study in healthy men found a 4-fold increase of MT protein in erythrocyte lysates, following supplementation with 15 mg of zinc for only 10 days. Plasma zinc was significantly increased between days 2 and 6 of the supplementation trial, and decreased thereafter. The monocyte and PBMC MT mRNA expression was significantly increased from day 2 to 12 in the supplemented group, compared to the placebo group (181). The researchers also obtained mRNA from dried blood spots equivalent to 50 μl whole blood.

The mRNA expression of MT followed a similar pattern as that of monocytes and PBMC. The sampling method is well described by the researchers and seems to be practical in the field; however, the blood spots need to be stored at -20°C and the filter papers have to be handled with special care (181). The above significant positive connection between MT mRNA as well as MT protein with dietary zinc intake was not supported by all researchers. For example, Sullivan et al. (1998) performed a placebo controlled supplementation trial, which was done over 18 days with 50 mg zinc/day in a group of healthy young men (baseline plasma zinc concentration of 11.8µmol/or 77.2 µg/dL). Both erythrocyte MT protein and monocyte MT mRNA were found to be useful in assessing increased zinc intake. But the researchers found no correlation between plasma zinc and monocyte MT protein. Importantly, the plasma zinc levels increased significantly at day 6 of the study and decreased to the baseline concentration at the end of the study (141).

The body loses about 5 to 10 mg zinc per day, approximately 50% of which is excreted through the feces. The feces contain unabsorbed dietary zinc, endogenous zinc from bile and from pancreatic fluid, as well as zinc contained in intestinal mucosa cells (2 to 5 mg/day). About 200 to 800 µg zinc per day is excreted in the urine. During pregnancy, the loss of zinc is reduced through the higher needs due to the growing placenta and fetus (11, 182, 183). Small amounts are excreted through hair and skin, the latter through exfoliation and sweating. The homeostasis between intake and excretion of zinc nomally makes up for reduced dietary intake by lower excretion (106, 183).

The body stores of zinc are minimal; only a few grams are stored in the bones and muscles. Most of the zinc is intracellular, mainly bound to MT. This makes MT an ideal

target to study zinc status. For example, in blood cells an increase of MT mRNA and protein was found due to zinc supplementation. At least half of the zinc is excreted through the feces, while the rest leaves via urine, sweat, or skin.

Assessment of zinc status

Prior to 1963, zinc deficiency disorders were not identified in humans (11). The global prevalence of zinc deficiency has only been roughly estimated due to the lack of reliable assessment methods (92). Future knowledge on the biochemical level will depend mainly on the development of assessment methods and adequate instrumentation (183).

Zinc is found intracellularly in all human organs, tissues and body fluids. However, to date, no simple biochemical analysis of zinc status is available. Further, mild to moderate zinc deficiency is difficult to detect with the available biomarkers (92). There are several ways to assess zinc in the human body. The assessment of zinc in red blood cells, leukocytes, neutrophils, and plasma is common. However, the zinc concentrations in these compartments are influenced by many factors unrelated to zinc status (143). For example, when the body's zinc needs increase, the commonly used plasma zinc may not follow linear patterns. The assessment of zinc during infancy and pregnancy is therefore very difficult, as the plasma zinc often does not correspond to growth patterns and supplementation with zinc does not always influence growth (184-186). Researchers found contradictory results in many studies of zinc status during pregnancy, because of the lack of adequate assessment methods that could account for non-linear increases due to hemodilution (187).

As another example of zinc assessment in different compartments, zinc was measured in fresh erythrocytes and plasma in order to assess the influence of inflammation and stress induced by a knee operation in otherwise healthy adults (60-84 years). Both parameters were assessed by inductively coupled plasma mass spectrometry (ICP-MS). Zinc decreased significantly by 40% in plasma within the first 24 h and stayed low until 168 h after the operation (188). As a result of an acute inflammatory reaction, albumin moves into the interstitial compartments and zinc bound to albumin would thus be removed from plasma (189). In erythrocytes, zinc concentration did not change significantly through the acute inflammatory response, which makes erythrocyte zinc a target compartment for zinc assessment (188). However, erythrocyte zinc was found to be elevated in hypothyroid hospital patients, compared to a healthy age-matched group. The reason for this elevation is unknown (190). A dietary zinc depletion-repletion study in 6 healthy young men revealed that urinary zinc decreased more rapidly than plasma zinc during the depletion period over week 4 to 11. Although individual decreasing patterns varied strongly, there was significant correlation between plasma and urinary zinc in all volunteers. While plasma, whole blood, urinary and fecal zinc decreased significantly, saliva, hair, and erythrocyte zinc did not change (191). Zinc supplementation trials have been used by several researchers investigating different aspects of health. Studies with healthy adults concerning cardiovascular disease revealed that not all subjects show increased serum or plasma zinc despite the supplementation levels (192).

Scientists are searching for alternative biomarkers like serum MT, zinc containing enzymes, urinary or hair zinc to assess zinc status in individuals. Each of these biomarkers has its advantages and disadvantages. Researchers investigated ways to

determine zinc in saliva by secretions as a measure of status beginning in the late 70's. Unfortunately, they found that plasma zinc did not appear to correspond with saliva measurements (193). Other studies revealed no correlation between zinc in saliva and hair compared to serum (13, 150, 193, 194). Even though higher levels of zinc were found in the saliva of men than in women, scientists think this is due to hydration, hormones, and other influences (13, 194). Decreasing amounts of zinc were found in both saliva and hair in the elderly (194). Similarly, low zinc concentration was found in the female subjects' saliva after a low zinc diet for 5 days (150).

The assessment of zinc in groups of the population can be achieved in different ways. Indirect methods can be applied in smaller children, for example, by measuring their height and comparing the results with growth standards based on expected height for age (195). A prevalence of more than 20 percent of stunting in children less than 5 years of age suggests a zinc deficiency problem for which nutritional intervention might be appropriate (196). However, growth is also affected by other nutrients and diseases. Next to anthropometric surveys, zinc status could also be estimated through dietary intake (zinc providing as well as inhibitory nutrients) and kinetic models employing national food balance sheets to calculate how much zinc is theoretically present to be eaten (196). Serum or plasma zinc have been sugested as population measures rather than an assessment of individuals, much like urinary iodine which is a measure for iodine levels of populations, not individuals (143).

Because of the limitations with reliable biomarkers for zinc, the global prevalence of zinc deficiency is only an approximation (92, 196). One of the problems with all zinc

sampling and assessment is possible contamination, for example in syringes, rubber lids of vacutainers, and powdered gloves, to name only a few handling errors (13).

Kinetic models with stable isotopes can help to estimate the uptake and distribution of zinc within the body. Models could contribute to the establishment of more precise intake recommendations. However, isotope studies are costly. In the past, signs of deficiency and toxicity were used to develop recommendations. Current innovative knowledge of nutritional research in dose-response curves explains deficiencies in one end and toxicity on the other end (196, 197)**.**

To conclude, although researchers have tried many approaches and have measured zinc in different sites, until today there is no non-invasive approach to measure zinc status in individuals. The comparison of results from zinc studies is difficult. Some researchers use supplementation trials, others depletion-repletion trials and the use of different concentrations of zinc and diverse populations make comparisons challenging. The currently used plasma and serum zinc assessments are more useful in measuring zinc status on a population or subpopulation level than on an individual level.

Novel assessment method for zinc

Although zinc was identified as important in human nutrition in 1964, assessing zinc status remains very difficult (11). Confounding results in the literature often originate from the lack of a verifiable method of assessment. In order to learn more about the metabolism of zinc, a reliable assessment method is needed (13, 92, 198). New technologies offer novel approaches to zinc assessment.

The quantitative real-time polymerase chain reaction (qRT-PCR) allows for a quantitative assessment of mRNA in cells. This allows for a very specific identification of mRNA in most compartments of the body. This method is highly specific and opens up new possibilities in the assessment field (18).

The genetic expression of Zip4 has been studied with these techniques in different models (Figure 5), for example in mice, where researchers demonstrated that dietary deficiency induced the expression of Zip4 mRNA but did not affect Zip5 mRNA levels (171). In enterocytes of a group of patients, the Zip4 protein diminished to undetectable levels after a zinc supplementation of 25 mg/d for 14 days, and in Caco-2 cells cultured in low zinc media (50% of normal levels), the mRNA expression was significantly reduced (173). Despite its importance in the uptake of zinc from the intestinal lumen, Zip4 was also found to have a negative role in the development of pancreatic cancer. Researchers found that a higher expression of Zip4 mRNA in pancreas tumors of mice was correlated to increased cancer proliferation, and consequently a higher mortality rate (199).

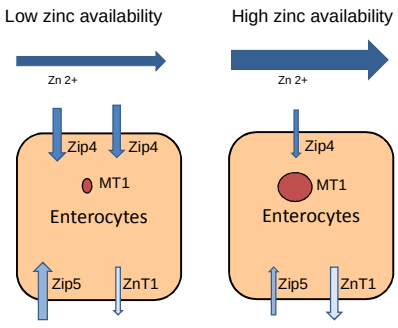

Figure 5: Principle of zinc transport in enterocytes, indicating low and high zinc availability in the intestinal lumen and effects on Zip4, MT1, Zip5 and ZnT1, adapted from Cousins et al. (70).

For the purpose of this study, the researchers tested a novel low invasive method of zinc assessment using buccal cells. The results were compared with the results of the current standard method for assessing plasma zinc. This new method is based on the assumption that a low zinc intake is correlated to a high expression of the zinc transporter Zip4 mRNA in buccal cells, and that an increase in dietary intake will lead to a decreased expression of Zip4 mRNA. The assessment of zinc would be easier if it were not necessary to draw blood. Therefore a method using buccal cells would mean little impact to the donor. The qPCR methods could identify changes in dietary zinc intake in several studies. However, the equipment and chemicals used for this study are very expensive and could not be used on a large scale or in limited resource laboratories.

Cortisol

Physiological function of cortisol

Cortisol, or hydrocortisone, is a major representative of the glucocorticoid family. Glucocorticoids are made from cholesterol through a series of enzymatic transformations in the adrenal cortex. Stressors affecting the hypothalamus trigger the release of corticotropin-stimulating hormone, which increases the release of adrenocorticotropic hormone from the anterior pituitary gland, which in turn increases the production of cortisol in the adrenal cortex. High cortisol in the bloodstream can impair the release of more corticotropin, creating a negative feedback loop (200).

Only about 10% of the cortisol in blood is free and therefore metabolically active, while the rest is bound to plasma proteins like the corticoid-binding protein (transcortin).

Free cortisol binds to the glucocorticoid receptors in the cytoplasm of all cells. Cortisol is transported to the nucleus and binds to a DNA glucocorticoid response element, modulating the transcription of several genes. For example, binding to a glucocorticoid response element inhibits the expression of many pro-inflammatory agents like cytokines, enzymes, and adhesion molecules. Anti-inflammatory drugs mimic this process (201, 200).

Cortisol plays an important role in metabolism by triggering the mobilization of proteins and fat as well as the breakdown of glycogen to be converted to glucose. As well, cortisol inhibits the uptake of glucose into muscle and fat stores and increases gluconeogenesis (200). A high circulating cortisol is sometimes seen as a sign of physiological stress (Figure 6). For example, the development of a tumor increases serum cortisol corresponding with the increased wasting process. Cortisol is most importantly strongly associated with the immunological status of a person (201). In general, cortisol is seen together with pro-inflammatory factors due to the fact that high blood cortisol levels are triggered by inflammatory cytokines. As well, immunological constraints like protein energy malnutriton or zinc deficiency are known to cause high serum cortisol (32, 202). Cortisol also has anti-inflammatory properties and glucocorticoids are often used as antiinflammatory agents. Cortisol induces zinc finger protein tristetraprolin (TTP) mRNA expression, and TTP is involved in the elimination of pro-inflammatory cytokines by binding to transcription factors and decreasing their transcription levels (32).

Important for this study is the fact that cortisol also seems to be connected to socioeconomic factors. Researchers found that an upbringing in a low socioeconomic environment was associated with higher cortisol and IL-6, as well as a resistance to

glucocorticoid signaling. These findings were independent of the socioeconomic status later in life. Prolonged increased levels of corticoid may lead to increased susceptability for chronic and inflammatory diseases later in life (203).

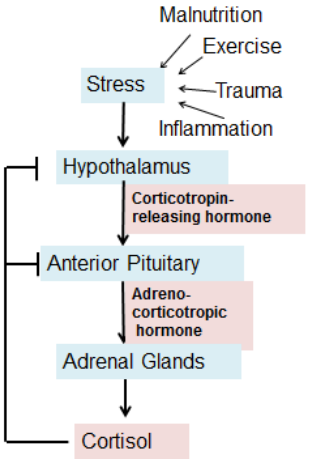

Figure 6: Effect of stress from different sources, such as malnutrition, exercise, trauma, inflammation, on the cortisol pathway via the HPA axis, adapted from Bowen, 2006 (200).

Important to the medical research and pharmacology interest is the fact that cortisol rises during physiological and psychological stress. For example, when the cortisol in adults is altered well above the reference of 4 to 28 nmol/L (1.5 to 10.2 µg/L), both blood tryptophan concentrations and brain serotonin concentrations decline. Stress causing low serotonin is acompanied by increased depression, and high pulse (204).

Diet can also influence cortisol concentrations. Current research indicates that stress- prone volunteers, when given a high protein and low carbohydrate diet, show high cortisol levels during and after stressful tasks, which were a battery of computer assisted tasks and tests . When given a high carbohydrate and low protein diet, the participants showed a strong trend for decreased cortisol ($p = 0.069$) and a significant decrease in all

the other stress markers, such as pulse rate and skin conductance. The lower cortisol was correlated to increased euphoria, security and self control. A second group of non-stress prone volunteers had with both diets increased cortisol levels after a stressful task (204).

In summary, cortisol is released from the adrenal glands in response to stressors triggering the hypothalamus-pituary axis. It inhibits proinflammatory agent genes. Cortisol also increases the breakdown of protein and fat stores for gluconeogenesis and contributes to the wasting process in chronic illness. high stress-prone individuals appeared to benefit from low protein and high carbohydrate intakes prior to a stress challenge.

Physiological cortisol levels

Cortisol levels in adults above 4 to 28 nmol/L (1.5 to 10.2 µg/L) indicated adverse health effects (204), such as impaired immunological function, and breakdown of muscle, fat, and glycogen (30). Fasting for one day increased serum cortisol 1.8 fold in healthy men, from 2504 nmol/L ± 308 to 4528 nmol/L ± 488 per 24 hours, measured at 5 minutes intervals and summed over the 24 hours time course of the study (205).

Assessment methods of cortisol

Cortisol is an important stress marker and can be used as a biomarker of physiological and emotional stress. It is an end product of glucocorticoid hormones from the hypothalamic-pituitary-adrenal axis (25, 26). Some studies indicate that cortisol in saliva and serum are closely correlated to each other. An increase of serum cortisol results in an increase of cortisol in saliva approximately 5 minutes later (36). The cortisol

in saliva represents the free cortisol, while around 90 % of the cortisol in the serum is bound to the cortisol binding protein. The threshold of cortisol binding in serum was found to be at 200 µg/L (551 nmol/L) meaning that free cortisol will be limited until the concentrations reach 200 ug/L in serum, and normal adult levels at 8 am are 60-230 µg/L (165-634 nmol/L) (36).

Furthermore, the use of saliva for cortisol measurments is comfortable for the volunteer and can be used for a series of chemical or enzymatic analyses (206). Because saliva contains only the biologically active form of cortisol and is easily collected at any time from the patient, saliva is an ideal screening compartment for cortisol levels. The early morning cortisol measurement is thereby the most accurate for whole day cortisol production (207).

There are several available enzyme based immunoassays (EIA) to assess cortisol in human samples. However, the Food and Drug Administration (FDA) has only recently cleared their use (REF). Several studies compared the established radioimmunoassay (RIA) methods with the relatively simple EIAs. An EIA for urinary cortisol was shown to have comparable results to the 24 hour urinary free cortisol RIA in Cushing's syndrome patients (208). Compared to the RIA serum total cortisol method, the EIA saliva samples showed similar results that were even more sensitive in their rapid response to changes in cortisol levels (35). The EIA in saliva needs a nearly tenfold smaller specimen volume. Furthermore, the incubation time was much shorter, while the range stayed about the same (2.8 to 276 nmol/L for EIA compared to 0.4 nmol/L to 276 nmol/L in the RIA). The EIA had a 1.7 fold higher recovery, than the RIA method, but EIA overestimated the recovery, while the RIA was closer to the actual cortisol concentration. The test

substance of 23 nmol/L cortisol was recovered as 22.4 nmol/L in the RIA (SD 0.7), while the EIA method had a recovery of 27.3 nmol/L (SD 0.6) (209). ELISA test kits for urine and plasma were found to be much faster than RIA methods, and results were comparable. Additionally, fewer waste products were produced with the ELISA method (210, 211). In general, ELISA methods are more sensitive than EIA, because the wash step in ELISA eliminates unbound specimens, which may interfere with the reaction in the EIA method. Both methods were developed in the 1960s and were first brought on the market in the 1970s (212).

The advantage of saliva as a specimen over 24-h urine and serum is that it can be gained easily in the field after minimal instruction. The patients can do the sampling by themselves, chewing on a polyester pad which fits into a test-tube. The sample does not need cooling until further analysis and can therefore be transported to the lab without any refrigeration. The sampling method and the fast, uncomplicated analysis through a readily available test kit make saliva an ideal method for testing cortisol in the field in rural Ethiopia.

In conclusion, cortisol can be measured in urine, serum and saliva. However, 90% of the serum cortisol is bound to a transport protein, which is thought to decrease its activity. Cortisol is highest in the morning and then decreases during the day. ELISA tests have replaced RIA and EIA tests in the cortisol literature. The measurement of saliva, furthermore, is increasingly chosen over urine or serum samples.

Interaction of zinc with cortisol

On a molecular level, thyroid hormones, steroid hormones, and zinc are connected. The first two are both part of the thyroid-steroid receptor family, a subfamily of the nuclear hormone receptor superfamily (213). Both hormones need zinc finger proteins in order to function. For example, in the case of an inflammatory reaction in the body, cortisol will be released from the adrenal glands. The released glucocorticoids bind to glucocorticoid receptors (GR), which contain zinc finger proteins, and which move into the nucleus and attach to glucocorticoid response elements (GRE). The connection of GR to the GRE starts the transcription of anti-inflammatory cytokines (eg. IL-1, IL-10). GR also inhibits the transcription of proinflammatory cytokines and chemokines by inhibiting the attachment of activated proinflammatory receptors to the respective response elements (214).

A study in mice indicated that zinc deficiency increased chronic stress and therefore cortisol. Elevated cortisol concentrations in turn diminished the T helper cell function and reduced thymus size and body weight. revealed that T helper cell function dropped, probably through the effect of zinc deficency, while the cortisol concentration subsequently increased (215). However, other studies showed clearly that the apoptosis in the thymus associated with zinc deficiency is not a direct effect of the zinc deficiency, but an indirect effect via increased cortisol levels (216). Chronically elevated cortisol concentrations due to zinc deficiency were responsible for increased cell death, especially in cells without the antiapoptopic Bcl-2 protein (217-219). These are mainly immature B and T-cells that drastically reduce the number of lymphocytes and therefore reduce the size of the thymus (220, 221). Apoptotic pathways, initiated by high cortisol

concentrations in conjunction with oxidative damage due to low zinc levels, lead to increased production of reactive oxygen species (ROS) and cytochrome c (functions also as intermediate of apoptosis). Both cause further damage to the cell and most importantly to the DNA (22, 80).

One supplementation study showed that increased dietary zinc reduced the production of cortisol very rapidly. After intravenous supplementation of a group of young men receiving 50 mg zinc, the plasma zinc content increased steadily from a mean of around 1 µg/mL up to 3.5 µg/mL within 110 minutes after administration, cortisol decreased from 14 µg/dl to 1 µg/dl in the same time span. In the same timeperiod, the cortisol concentration of the control stayed in the narow range of 8-11 µg/dl. The same pattern was found in the groups receiving 25 and 37.5 mg zinc, however, both had their highest zinc and lowest cortisol level at around 200 minutes, compared to 110 minutes with the 50 mg zinc dose, demonstrating the inhibitory effect of zinc on cortisol (24). However, another supplementation (30 mg zinc) trial over 6 months did not have a significant effect on the cortisol concentration of diabetic children. Plasma zinc was somewhat higher at three months, but only after 6 months did the supplemented group show significant increases (222).

In conclusion, zinc finger motifs are important elements in molecular signaling. Therefore zinc is a central factor in pro- and anti-inflammatory actions. Some studies show that decreased zinc concentrations were associated with increased cortisol in the body. While increased cortisol levels cause cell death in T- and B-cells, leading to thymus atrophy. In conjunction with a decreased antioxidant defense due to low zinc levels, elevated cortisol leads to increased production of ROS. But also increase zinc

levels may have a negative effect on cortisol. A study indicates that an injection with zinc lead to decreased plasma cortisol concentrations within minutes.

CHAPTER III

MATERIALS AND METHODS

Location and study design

Study area and population

For this study, the village of Finichawa in the Sidama region was chosen, based on participation in a previous study. The village is 20 minutes by car from Hawassa University in Awassa, lies at about 2000 meters above sea level, and has about 5000 inhabitants. Finichawa has access to roads but is mainly an agricultural community. The research unit in the Institute of Nutrition, Food Science and Technology of Hawassa University was responsible for the collection and handling of the samples.

The sample size was chosen based on similar zinc supplementation studies, which had significant plasma zinc changes with small groups and short duration of supplementation (223, 224). However, the original supplementation period of 21 days had to be shortened to 17 days, a shorter time period than the reference studies. This was due to time constraints of the field investigator.

After the first cross-sectional study by Tafere G/Egziabher, with 200 participating women in 2009, a group of volunteers in the Finichawa village were asked if they would participate in a second study. Women aged 18 to 45 years in good health were included in the study. A local health worker assessed the health status of the women. In addition to

impaired health, a further exclusion criterion was self-reporting of pregnancy. The volunteers were informed of the trial ahead of time. The day before the trial, the women again received oral information about the trial protocol from the local staff. If they decided to participate, the women were asked for a verbal consent, which was witnessed by a community member. The next day the women returned to begin participation. A convenience sample of forty volunteers 18 years or older was randomly split into two groups of 20. The women received a blanket in appreciation for their participation.

Ethical Approval

The approval process for this project started in August 2007 and was finalized in July 2009. The research had to be approved at Oklahoma State University (OSU) and Hawassa University, as well as at the regional and national level in Ethiopia. The study was approved by the OSU Institutional Review Board (Appendix A and B), and the Health Research Ethics Committee of Hawassa University (Appendix C). Furthermore, approval was granted on a regional level from the South Nations Nationalities and People's State Health Bureau (Appendix D) and the Health Office of Awassa (Appendix E). On the national level, approval was granted by the National Health Research Ethics Review Committee at the Ethiopian Science and Technology Commission (Appendix F) and the Drug Administration and Control Authority (Appendix G).

This research was supported by the Swiss National Science Foundation (PBSKP3-124358), NIH Grant R01HD053053 (NICHD & Fogarty International Center), Hawassa University, Oregon State University, and Oklahoma State University.

Study design

This study was a double-blind placebo-controlled zinc supplementation trial involving 40 women from the same village in Ethiopia. The supplementation of 20 mg zinc as zinc sulfate per day was given to the twenty women of the treatment group, while the twenty members of the other group received a placebo.

Pre-survey Preparations

Several methods were tested in the laboratory at Oklahoma State University. However, the use of RNA later was not evaluated prior to sampling. The material was shipped by an international carrier to Hawassa University, which took an average of 3 days. Samples had to be obtained rapidly in the field and then transported to Hawassa University. The contact at the Ethiopian Health and Nutrition Research Institute served well in this matter and assisted the researchers in the making of dry ice for transportation. Several researchers and staff members of Hawassa University worked on this trial.

A written protocol for the testing procedure was developed to guide the local researchers through the sampling process. Questionnaires were administered and samples collected by researchers from Hawassa University, assisted by local community workers hired by the research unit. Dr. Barbara Stoecker assisted with field sampling and transportation of the samples back to OSU's Stillwater campus for biochemical analysis, while Dr. Stephen Clarke supervised the laboratory work.

Zinc supplements and placebo

Supplements containing 20 mg zinc as zinc sulfate or placebo (ZinCfant, Nutriset S A S, Malaunay, France) in dispersible tablet form were given to the volunteers as two tablets each morning for the 17 days of the trial. The zinc supplements were thus well below the upper level of 40 mg/d (102).

Sampling and data collection

The zinc supplements as well as the placebo were distributed to the volunteers at the local health center every morning. The volunteers took two tablets daily in the health center with water provided to them. The total supplement was 20 mg zinc in the form of zinc sulfate or a placebo. The supplementation period started on January 7th, 2010, which was day 1 of the 17 day supplementation. On the first and the 18th day of the study, the blood, saliva, and buccal cell samples were obtained.

The questionnaires were completed on day one by individual interview in the local language, Sidaminga, because most women had little or no formal education. On the same day, height and weight of the volunteers were measured twice, standing upright and wearing light clothing. Height (m) and weight (kg) measurements were taken as described by WHO, 2006 (225). The body mass index (BMI) was calculated by dividing the weight in kg by the height in m^2.

For the venipuncture, sterile, disposable syringes and 21 gauge needles (Sarstedt, Newton, NC) made from trace-mineral free plastic were used by a trained laboratory technician. In brief the urine samples were collected in cups and transferred to small vials for storage. The buccal cell samples were obtained with a toothbrush and saline solution

(0.9% NaCl) in 50 mL centrifuge tubes. The samples were centrifuged and the cells recovered. Saliva samples were obtained with a polyester pad (Salivette, Sarstedt, Newton, NC). Further sampling methods are described below.

The collected samples and planned measurements are listed in Table 1.

Table 1: List of samples collected and planned analyses.

Samples	Products of samples	Substance Analyzed	Method
Saliva	Saliva	Cortisol	ELISA kit
Buccal cells	Buccal cells with RNA later	Zip4, MT	PCR
Li-heparinized blood	Plasma	Zinc	ICP-MS
	Washed red blood cells	Zinc	ICP-MS
	Whole blood	Comet assay	Electrophoresis
	Whole Blood with RNA later	Zip4, MT	PCR
	Leucocytes with RNA later	Zip4, MT	PCR
Non- anti-coagulated blood	Serum	Cortisol	ELISA kit
		α-1 Acid Glycoprotein	ELISA
		C-reactive protein	ELISA kit
		Albumin	Clinical method
		FRAP	Clinical method
Urine	Urine	Iodine	Clinical method
		Cortisol	ELISA kit
		Total Protein	Clinical method
		Creatinine	Clinical method

Biochemical analysis

Zinc in plasma

Blood samples were drawn into heparinized plastic tubes certified for trace-element research that were purchased from Sarstedt (Sarstedt, Newton, NC). Samples were stored on ice, centrifuged, and separated into plasma and packed erythrocyte fractions within two hours. Plasma samples were stored at -20°C until they were transported on dry ice to the laboratory at Oklahoma State University, where they were stored at -80°C.

For analysis of plasma zinc, 200 µl of plasma, 8 µl of internal standard (Gallium 2%, Perkin Elmer Life and Analytical Sciences, Shelton, CT) and 3.8 mL of 0.1% HNO_3 (GFS Chemicals, Powell, OH) containing 0.01% Triton X (octyl phenoxy polyethoxyethanol, T-6878 Sigma-Aldrich, Inc, St. Luis, MO) in Millipore water were mixed. This 20-fold dilution was assessed by ICP-MS using a Perkin Elmer SCIEX Elan 9000 (Perkin Elmer Life and Analytical Sciences, Norwalk, CT), using external serum standards (Utak Laboratories Inc., Valencia, CA) for quality control. The method assessed copper and selenium together with the zinc levels. Healthy women 20 years and older were suggested to have plasma or serum zinc concentrations at 700 µg/L and above obtained in the morning after an overnight fast (226).

Zinc in erythrocytes

Erythrocytes obtained from Li-heparinized blood while gaining plasma were washed in the laboratory at Hawassa University with a 0.9 % saline solution in an amount

about three times the volume of the erythrocytes. The erythrocytes were carefully mixed with the saline solution and then centrifuged at 350 x g. The supernatant was discarded and the wash step was repeated three times. The erythrocytes were frozen at -20°C and transported on dry ice to Oklahoma State University.

To assess erythrocyte zinc levels, new borosilicate glass tubes were acid washed (soaked in 1 M HCL, then rinsed with distilled water, and finally rinsed with Millipore water) and dried in the oven, and then 35µl of the erythrocytes were placed into the tubes. The samples were then wet ashed with 50 µl of double-distilled nitric acid (69.7%, GFS Chemicals, Powell, OH), 50 µl hydrogen peroxide (M-341, 50%, Thermo Fisher Scientific Inc., Hanover Park, IL) and 50 µl Millipore filtered water (Milli-Q Direct, Billerica, MA) in a heat block at 95°C. The wet ashing chemicals were added 5 times, and then the samples were dried. After this the samples were dry-ashed in a muffle furnace for 24 hours at 375°C, raising the temperature gradually 5°C per minute after starting at room temperature (Lindberg/Blue, Asheville, NC). The samples were then wet ashed five more times with the above mentioned chemicals and then dried. The measurement in the ICP-MS was performed according to the plasma zinc protocol above. In the literature, normal adult erythrocytes ranged from 10.4 to 15.1 mg/L zinc, as measured by atomic absorption spectrophotometry (227).

Zip4 transporter and metallothionein in buccal cells

For the novel indirect assessment method of zinc, buccal cells were collected with an adapted method developed by Liu et al. (228). Photo images of the different steps were provided to lead the women through the sampling. The mouth was rinsed twice with

clean water and then buccal cells were brushed off the inside of the cheek with a new, individually wrapped tooth brush. Finally, the mouth was rinsed with 20 ml of a saline solution (0.9%) which was collected in a tube after the subjects rinsed their mouth. The toothbrush was rinsed in the collected solution for sample recovery. In the field the samples were stored on ice. Upon arrival in the laboratory at Hawassa University, the samples were centrifuged at 350 x g and the supernatant was discarded. The 1.2 ml of RNA later solution (AM 7023, Ambion, Inc., Austin, TX) was then added to the buccal cells. The samples were initially stored at room temperature and transported at -20°C to the laboratory in the U.S., where they were kept for another week at -20°C and finally stored at -80°C.

In the laboratory at Oklahoma State University, some samples were extracted with TRIzol Reagent (Invitrogen, Ambion, Inc, Austin. TX), according to the provided protocol. Thereafter, the RNA of the samples was extracted with TRIzol Reagent and chloroform (BDH, VWR Inc. West Chester, PA). The RNA was then precipitated using isopropyl alcohol (ACS grade, Pharmco-AAPER, Brookfield, CT). Finally, the RNA was washed with ethyl alcohol (75%, USP grade, APPER Alcohol and Chemical Co., Shelbyville, KY).

The RNA concentration was measured with the use of a UV spectrophotometer (Nano Drop ND-1000, Thermo Fisher Scientific Inc., Waltham, MA) at A260 and A280, expressed as A260/A280. In addition, the RNA quality was assessed through electrophoresis with Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA). A sample of 1µg of RNA was used to generate a double stranded cDNA with Super Script III Reverse-Transcriptase (Life Technologies, Carlsbad, CA) in a thermocycler

(Thermocycler Biometra TGradient, Biometra GmbH, Goettingen, Germany).

The zinc transporter Zip4 and the MT mRNA expression of the samples were detected at baseline and at endpoint of the study by quantitative Real-Time PCR (7900 HT Fast Real-Time PCR System, Applied Biosystems, Foster City, CA). The 25 nmole DNA Oligonucleotide primers (19 bases) (Integrated DNA Technologies, Inc., Coralville, IA) were used (Table 2).

Table 2: List of the primers for Zip4, MT1, and the housekeeping gene β-actin with accession number, sense, and antisense primers used for this study.

Gene	Accession number	Sense Primer	Antisense Primer
Zip4 / SLC 39A4	NM_130849.2	5’ AAT AGG GAC GAT TGC CTG AT 3’	5’ GCC AGG CCA TCG AAA T 3’
MT1	NM_005946.2	5’ CTC CTG CTG CCC CAT GAG 3’	5’ TCT GAT GCC CCT TTG CAG 3’
β actin	NM_001017992.2	5’ GCA CCC AGC ACA ATG AAG AT 3’	5’ GCC GAT CCA CAC GGA GTA C 3’

Cortisol in saliva, serum, and urine

The participants were asked to provide a sample of their saliva for the cortisol determination. Each woman chewed a polyester pad stick (Salivette, Sarstedt, Newton, NC) that filled with saliva. The sticks were then transferred into their specific tubes and stored on ice. In the laboratory, the test tubes were centrifuged and the saliva transferred to the storage vials. The saliva was frozen and transported on dry ice.

For the cortisol assessment, an ELISA test kit for total cortisol (Parameter Cortisol, R&D Systems, Inc. Minneapolis, MN) was used. The samples were diluted two-fold, not the five-fold dilution as suggested by the manufacturer, because a five-fold dilution lowered many samples below the minimum detection range. The analysis was

performed according to the manufacturer's recommendations. The principle of the analysis was a competitive ELISA in which cortisol in controls and serum bound to mouse monoclonal antibodies immobilized in the wells. The cortisol in the specimens bound to the immobilized antibodies. After a wash step, horseradish peroxidase (HRP) labeled cortisol was added, which bound to the remaining antibodies. A further wash step was necessary in order to remove the excess cortisol. A substrate solution was added to measure the enzyme activity through color development. The absorbance was measured at 450 nm in a plate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT). Quality control sera (RD6 for Cortisol, R&D Systems, Inc., Minneapolis, MN) were used to assess the validity of the assay for low (0.68-1.33 ng/mL), middle (1.99-3.76 ng/mL), and high (3.74-5.95 ng/mL) cortisol samples.

According to the manufacturer, mean minimum detectable concentration in thirty-one assays was 0.071 ng/mL. Healthy volunteers had saliva cortisol levels between 0.76 to 2.94 ng/mL with this kit. In the literature ranges of 0.94 to 15.14 mg/mL (2.6 to 41.8 nmol/L) can be found for healthy adult women, aged 31 to 40 years (229). The sample concentrations were interpolated based on the calculated controls using a four parameter logistic curve fit (GraphPad Software, Inc., La Jolla, CA).

For the assessment of serum cortisol, the same test kit as for saliva cortisol was employed. The samples were diluted twenty-fold. The cortisol concentration of 35 healthy volunteers ranged from 16.8 to 75.5 ng/mL using this ELISA plate (Parameter Cortisol, R&D Systems, Inc., Minneapolis, MN).

Urinary cortisol was measured with the above mentioned kit. The urine samples were twenty-fold diluted. The range for 13 healthy adults was 20.8 to 153 ng/mL using this ELISA plate (Parameter Cortisol, R&D Systems, Inc., Minneapolis, MN).

Iodine in urine

Urinary iodine was measured with a modification of the Sandell-Kolthoff reaction (230), using ammonium persulfate for the digestion (231). Thereafter, iodine was reduced by cerric ammonium sulfate, which is yellow to the colorless cerrous ion. The reaction was coupled to the oxidation of arsenous acid from As^{3+} to As^{5+}. The method was performed with 60.75 µl of urine, which was digested with 250 µl of 1 M ammonium persulfate at 95°C for 60 minutes. To the cool samples, 600 µl arsenous acid was added, and the mixture was incubated for 15 minutes before the addition of 75 µl of cerric ammonium. The samples were measured exactly 30 minutes later at 405 nm in a spectrophotometer (Beckmann spectrometer DU 800, Fullerton, CA).

Urinary iodine levels are measured in order to assess community levels of deficiency, rather than the iodine status of a single person, because iodine varies widely during the day. The iodine status of the volunteers was reported as the percentage of participants in each of the six categories defined by the WHO: median urinary iodine of < 20 µg/L (severe iodine deficiency), 20-49 µg/L (moderate deficiency), 50-99 µg/L (mild iodine deficiency), 100-199 µg/L (adequate iodine nutrition), 200-299 µg/L (above requirements), and >300 µg/L (excessive risk for adverse health effects) (232). However, the two highest categories will not be used for this research.

Ferritin in serum

For the assessment of serum ferritin, an immunoradiometric assay (IRMA F-11, Ramco Laboratories, Inc., Stafford, TX) was performed. For this assay, a radiolabeled antibody was added to the 100-fold diluted serum; the antibody binds to the ferritin in sample and controls. Next, beads coated with rabbit antibodies against human ferritin were placed in tubes containing antibody plus calibrator, control, or serum. After the beads were added, the tubes were incubated for two hours on a shaker to allow for full contact with the beads, so that ferritin could attach to the the antibodies on the beads. Tubes were then incubated overnight. The next day, the liquid phase was aspirated, the beads were rinsed tree times with deionized water, and each tube with its bead was quantified in the gamma counter (Cobra II Auto Gamma Counter, Packard, Downers Grove, IL, USA) for 2 minutes. The calibration curve was calculated according to the manufacturer's suggestion using a log transformation for the concentration and a linear scale for the counts (GraphPad Software, Inc., La Jolla, CA). Ferritin concentration in serum and controls was interpolated based on the standard curve. Iron deficiency (ID) was defined as ferritin concentrations of <15 μg/L (233).

Comet assay in whole blood

Alkaline single cell gel-electrophoresis was used to assess single-strand breaks (234). For the comet assay, whole blood was collected into Li-heparin coated tubes and stored at 4°C in the field and during transportation. The method used for sample preparation is described by Song et al. (23). Due to slight hemolysis, the blood samples were centrifuged, and the cell sediment was used for the comet assay instead of

performing the common wash step. The cell material was embedded in low melting agarose (5%, BP165-25, Thermo Fisher Scientific Inc., Hanover Park IL) dissolved in phosphate buffered saline (100-3, Sigma-Aldrich, Inc., St. Louis, MO). Fifty µl of the agarose containing the cells was pipetted, spread onto each of the three well slides (3950-300-02 FLARE Slides, Trevigen Inc., Gaithersburg, MO), and then treated with lysis solution (10% DSMO, Sigma-Aldrich, Inc., St. Louis, MO) to unwind and denature for one hour. The slides were placed into the electrophoresis chamber filled with alkaline buffer (0.3 M NaOH/1 mM EDTA) and stored for 20 minutes. The electrophoresis was run for 25 minutes at 25 V and 300 mA. After the electrophoresis, a neutralization buffer (0.4 M Tris pH 7.5, TRISMA Base, T-6066, Sigma-Aldrich, Inc., St. Louis, MO) was applied to the slides three times for 5 minutes each. After this, the slides were submerged in cold methanol (100%, 339000DIS, Pharmco-AAPER, Brookfield, CT) and ethanol (100%, 459828, Sigma-Aldrich, Inc., St. Louis, MO). The slides were then stored until analysis at Oregon State University under the supervision of Dr. Emily Ho, as described by Song et al. (23). The assessed measurments were tail length, tail moment, tail intensity, total area, total intensity, and width.

α-1 acid glycoprotein in serum

To assess the acute phase protein α -1-acid glycoprotein (AGP), an ELISA quantification kit (40-288-22927F, Gen Way Biotech, Inc., San Diego, CA) was employed. Therefore, 96-well plates were coated with 200-fold diluted capture antibodies for 60 minutes, and the excess antibodies were removed in a wash step. A blocking solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) was added to the wells, which

were then incubated for 60 minutes, followed by a wash step. Then calibrators, serum, and controls (dilution 1:8503) (Kentrol Reference serum, Kent Laboratories, Inc., Bellingham, WA) were added to the wells. To build a sandwich with the immobilized antibody and antigen, a second antibody was added containing horseradish peroxidase. After an incubation of 60 minutes, the wells were washed five times. An enzyme substrate was added exactly 40 minutes later, and the reaction was stopped with sulfuric acid (2 M, 290000ACS, Pharmco-AAPER, Brookfield, CT). The 96-well plate was then read at 450 nm in a plate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT).

The standard curve was calculated with a four parameter logistic curve fit (GraphPad Software, Inc., La Jolla, CA). The AGP in serum and controls were interpolated based on the standard curve. The detection range of AGP test kit was 2.74-2000 ng/mL. The controls lay within the expected range. Levels above 1.2 g/L indicate inflammatory processes (235, 236).

High sensitivity C-reactive protein in serum

High sensitivity C-reactive protein (CRP) is produced in the liver in acute conditions of trauma, infection, or inflammation. For this analysis, an ELISA test kit was used (961CRP01H, Helica Biosystems, Inc., Fullerton, CA). The 96-well plates precoated with rabbit anti human CRP antibodies were used; standards, as well as controls and serum samples (diluted 1000 fold) were added to wells in the plate. After a wash step, horseradish peroxidase labeled rabbit anti human CRP antibodies were added to bind with the immobilized antibody and antigen. After a further wash step, urea peroxide substrate containing tetramethyl benzidine (TMB) was added as a chromogenic substrate.

The solution turned blue, and after 10 minutes the acidic stop solution was added and the solution became yellow. The optical density was assessed at a wavelength of 450 nm in the plate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT). A standard curve was created which was used to interpolate the sample and control concentrations. The controls (Kentrol Reference serum, Kent Laboratories, Inc. Bellingham, WA) lay within the expected ranges of 0.1 to 10 µg/mL (0.1-10 mg/L). In 59 healthy volunteers serum CRP concentrations below 3.8 µg/mL (3.8 mg/L) were measured with this test kit (961CRP01H, Helica Biosystems, Inc., Fullerton, CA), and the kit has a detection limit of 0.20 ng/mL. The cutoff for high CRP was assumed to be 3 mg/L for this study (237).

Ferric reducing antioxidative power measured in serum

The ferric reducing antioxidant power (FRAP) assay, sometimes called ferric reducing ability of plasma assay, is modified from the method developed by Benzie and Strain in 1996. Briefly, the antioxidant activity in samples is measured by their ability to reduce ferric tripyridyl triazine (Fe III TPTZ) to the ferrous form (Fe II) at a low pH. The ferrous form of iron is blue and can be measured in the spectrophotometer at a wavelength of 593 nm. The method is not specific to any single serum antioxidant (ascorbic acid, uric acid, bilirubin, α-tocopherol, etc.); instead the total antioxidative capacity of the samples is measured (238).

For the FRAP measurement, a 10:1:1 mixture of acetate buffer pH 6 (300 mM, sodium acetate trihydrate S-9513, Sigma-Aldrich, Inc., St. Louis, MO, dissolved in glacial acid, 281000ACS, Pharmco-AAPER, Brookfield, CT), 2,4,6-tripyridyl-s-triazine (10 mM TPTZ, 70204-1EA-R , Sigma-Aldrich, Inc., St. Louis, MO, dissolved in 40 mM

HCl, H1035, Spectrum Chemical Mfg. Corp. New Brunswick, NJ) and iron chloride (20 mM $FeCl_3$ *6 H_2O, 1-1996, J. T. Baker, Phillipsburg, NJ) was produced. Iron sulfate ($FeSO_4$, FX265, MBC Manufacturing Chemists, Inc., Cincinnati, OH) served as standard. One hundred and fifty µl of the working reagent was pipetted into 96-well plates, and the absorption at wavelength 593 nm was measured in a plate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT). Twenty µl of standard or serum was added to the wells. After shaking the plate well, it was covered and stored at 37°C for 8 minutes. Again the optical density was measured at wavelength 593 nm. The difference between the two optical densities was calculated. A linear calibration curve was drawn from the standards, which served as the base to interpolate the serum concentrations. Healthy adults had FRAP results of 600 to 1600 µmol/L. The detection limit of the method is < 2 µmol/L (239).

Albumin in serum

Serum albumin was determined with an albumin reagent set (BQ 002 CR, BioQuant Diagnostic, San Diego, CA). Albumin binds at acidic pH to the dye bromocresol green. The color difference between the reagent and the serum-reagent mixture is proportional to the albumin concentration and can be measured at 630 nm.

The standards from 0 to 7 g/dL albumin were prepared. Standards, control (GEMCAL Reference Serum, Alpha Wassermann Diagnostic Technologies, LLC., West Caldwell, NJ), and samples were diluted two fold for the analysis. Of the specimens, 3.34 µl were pipetted into 96-well plates and 250 µl albumin reagent added. After exactly 5 minutes at room temperature the samples were measured in a plate reader at an optical

density of 630 nm (Synergy HT, BioTek Instruments, Inc., Winooski, VT). Albumin concentrations of the controls lay within the expected levels. The method had a lower detection limit at albumin concentrations of 0.005 g/dL. Normal levels are between 3.5 and 5.3 g/dL (240).

Total protein in urine

For the assessment of total protein, the bicinchoninic acid (BCA) method (total protein test kit (PI 23227, Thermo Fisher Scientific Inc., Hanover Park IL) was used. Urine samples and controls (GEMCAL Reference Serum, Alpha Wassermann Diagnostic Technologies, LLC., West Caldwell, NJ) were diluted tenfold and seventy fold respectively. The first step of the reaction is the biuret reaction. The peptides with three or more amino acids and cupric ions (Cu^{2+}) form a blue chelate, reducing cupric ions into cuprous (Cu^{1+}) in alkaline solution containing sodium potassium tartrate. In the second step of the reaction, two ions of bicinchoninic acid bind to one cuprous ion, creating a purple color which can be measured after an incubation time of 30 minutes at wavelength 562 nm in a plate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT). The controls lay within the expected range. Working range of the method is 10-2000 µg/mL. For the assessment of total protein in urine the method described for total protein in serum was used. The urine samples were diluted ten-fold. Normal ranges are 10-80 mg/L.

Statistical Analysis

For the descriptive statistics, the means, medians (CRP, iodine) standard deviations (SD), and standard error of the mean (SEM) were calculated in SPSS/PASW (PASW Statistics 18.0, IBM Corporation, Somers, NY).

The effects of the zinc supplementation in both groups (supplemented and placebo) at baseline and endpoint were assessed with a two-factor within-subjects ANOVA using SPSS/PASW (PASW Statistics 18.0, IBM Corporation, Somers, NY) (224). The data were checked for normality prior to analysis. Skewed data (serum ferritin, serum C-reactive protein) (cut off more than 3.0 standard deviations away from the mean) underwent square root transformation. The data was expressed as mean ± SEM, and an alpha error level of $p < 0.05$ was set (241).

For the comet assay, treatment effects were analyzed using analysis of variance with repeated measures (PROC MIXED) with an autoregressive period 1 error structure. The simple effect of treatment given time (SLICE option in an LSMEANS statement) was used to identify significant changes from baseline with supplementation (SAS Institute, Inc., Cary, NC, USA).

For the Pearson's correlation coefficients, the statistic software SPSS/PASW (PASW Statistics 18.0, IBM Corporation, Somers, NY) was used.

CHAPTER IV

ASSESSMENT OF CHANGES IN mRNA EXPRESSION IN BUCCAL CELLS AT BASELINE AND AFTER A PLACEBO-CONTROLLED ZINC SUPPLEMENTATION TRIAL IN SIDAMA, ETHIOPIA

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Abstract

Limitations in the use of plasma or serum zinc for assessing changes in zinc status, in conjunction with newer assessment methods targeting changes in mRNA expression, have increased the impetus in the field of zinc research to develop improved zinc assessment methods in humans. In this study, the use of the zinc transporter ZIP4 (Slc39a) mRNA and the metal binding MT1 mRNA in buccal cells were investigated as measurements for the change in zinc intake.

First, a method was refined based on established methodologies to harvest sufficient quantities of buccal cells from which RNA could be extracted. Following total RNA extraction, cDNA was synthesized to examine zinc-dependent alterations in gene expression by quantitative real-time PCR (qPCR). Initial results in a pilot study

conducted in a laboratory environment suggested that expression of ZIP4 mRNA was repressed in response to oral zinc supplementation; however, no significant correlation could be established between expression levels of ZIP4 mRNA and plasma zinc concentrations. Unfortunately, we were unable to further test our hypothesis with samples obtained from the placebo-controlled trial due to poor RNA extraction and quality of samples collected in the field.

Despite technical difficulties with sample collection and extraction, the results from present studies suggest that examining zinc-regulated gene expression using a relatively non-invasive approach could prove a viable option for assessing zinc status in individuals. Interestingly, we were unable to detect a correlation between the expression of zinc-regulated genes and plasma zinc concentrations in the pilot study containing healthy (i.e., zinc-sufficient) volunteers. Based on these preliminary results, we suggest that plasma zinc levels may not be an appropriate reference method to compare the results of the qPCR analyses. Furthermore, plasma zinc concentration and zinc-regulated gene expression may not necessarily be reflective of daily alterations in zinc intake. Additional strategies should be employed to maximize the non-invasive collection and stability of buccal cell RNA for future gene expression analyses from community-based trials.

Introduction

Severe zinc deficiency is widespread among developing nations. Even among more developed nations, mild to moderate zinc deficiencies remain relatively common and prevalent especially in vegetarians and elderly (1). In developing countries, areas

with a mainly plant-based diet, low in meat and dairy are the most prevalent for zinc deficiency. In Sidama, Southern Ethiopia low meat consumption combined with a high phytate and fiber intake contribute to the incidence of zinc deficiency. Examining only total dietary zinc intake may not fully account for alterations in zinc bioavailability, which can be impaired by other dietary factors such as phytate and calcium (2). A study in 99 pregnant women found that ~72% were below 700 µg/L zinc in plasma, the lower end of the intake recommendation for adults. In addition to zinc deficiency, many individuals in this region of Ethiopia have marginal intakes of energy, protein, iron, and calcium (3, 4).

Both plasma and serum zinc are the most widely accepted methods of assessing zinc status, but these pools of zinc may be influenced by factors independent of dietary intake. To further complicate assessment of zinc status, the majority of zinc is found intracellularly suggesting that measurements of plasma or serum zinc may not accurately reflect tissue concentrations (5). Although plasma and serum zinc levels are useful biomarkers of zinc status within a population, the extent to which plasma and serum zinc levels are indicative of zinc status among individuals remains controversial (6). Due to these discrepancies, our group is interested in developing new or modifying existing methodologies that are compatible with limited sample availability yet are sensitive measures of zinc status. Ideally, these methodologies would increase our ability to determine an individual's zinc status using either direct or indirect approaches. Though relatively new to field-assessment strategies, technologies like the exquisitely sensitive quantitative real-time polymerase chain reaction (q-PCR) could offer innovative approaches for assessing nutrient status at both the individual and population levels.

Quantitative PCR allows investigators to determine the relative abundance of mRNA among a variety of samples. Provided the sensitivity of the method, qPCR-based strategies expand the possibilities in the nutrition-assessment research field. For example, qPCR allows for the identification and quantification of zinc-regulated genes such as the zinc transporter ZIP4 (Slc39a4) (7). In humans ZIP4 is primarily expressed in the apical site of enterocytes, pancreatic β-cells of the islets, and endoderm cells of the yolk sac, where changes in dietary intake affect the expression of ZIP4 mRNA (8-10). In both animal models and human studies, dietary zinc deficiency increased ZIP4 mRNA expression in intestinal cells, whereas zinc supplementation decreased it (10, 11).

In addition to zinc transporter ZIP4, the metal chelator metallothionein (MT) was one of the target cell substances of this study. MT, a cysteine-rich protein located in the membrane of the Golgi apparatus, has the ability to bind metals. These metals are on one side physiological (copper, zinc, iron) and on the other xenobiotic (cadmium, mercury, silver, arsenic), which are bound through the thiol group of its cystein residues (12). During zinc supplementation, MT mRNA expression increased significantly in leukocytes extracted from dried blood spots (13, 14). In cell culture models increases in MT mRNA was associated with increases in MT protein abundance (14). Tests in lymphocytes and monocytes showed that MT mRNA was even more sensitive than the MT protein towards dietary changes (15).

The goal of this study was to assess the mRNA expression of Zip4 transporter and MT1 in buccal cells were assessed at both time points with the qRT-PCR method. A correlation between both measurements was investigated in buccal cells. Furthermore, the

changes in plasma zinc concentration at baseline and endpoint of a placebo-controlled zinc supplementation trial.

Volunteers, materials and methods

Volunteers Stillwater, OK

To conduct a small preliminary trial, a group of students and staff members at Oklahoma State University (OSU) was recruited. The volunteers signed a written consent and provided a buccal cell sample and Li-heparin blood samples drawn into trace-mineral free EDTA coated tubes. The blood was immediately separated (350 x g for 20 minutes) and then the plasma was stored at -20°C. The buccal cells were centrifuged at 350 x g for 20 minutes and then separated from the supernatant. The cells were then stored at -20°C.

Volunteers Finichawa, Sidama region, Ethiopia

Forty non-pregnant women, age 20 to 45 years, from the rural Finichawa village in the Sidama region of Ethiopia participated in a placebo controlled zinc supplementation trial. The day before providing oral consent, the women attended an orientation session by local staff from Hawassa University (located nearby in Awassa, Ethiopia), explaining the goal of the study and the consequences of participation. A health worker excluded women with signs of health impairment from the study.

Study design, supplementation, sample collection (Finichawa, Sidama region, Ethiopia)

Half of the chosen women randomly received a supplement of 20 mg zinc in the form of zinc sulfate (ZinCfant, Nutriset S A S, Malaunay, France), while the other half

took a daily dose of placebo (Nutriset S A S, Malaunay, France) for 17 days. At day 1 and at day 18 the volunteers provided blood and buccal cell samples. For the blood draw, trace-mineral free sterile lithium-heparin coated plastic tubes were used (Sarsteadt, Newton, NC). Plasma was immediately separated from erythrocytes and kept on ice in the field and at -20°C at Hawassa University. The buccal cells were obtained following a protocol with pictures adapted from a method developed by Liu et al. (16). The buccal cell samples were kept on ice in the field. At Hawassa University, buccal cells were separated by centrifugation from the saline solution with which they were washed out of the mouth. Buccal cells were stored with RNAlater (AM 7023, Ambion, Inc., Austin TX) and stored at room temperature for one day. The samples were kept at 4°C until the end of the study (day 18) and then transported at 4°C to the laboratory at Oklahoma State University.

For the Pearson's correlation coefficients, the statistic software SPSS/PASW (PASW Statistics 18.0, IBM Corporation, Somers, NY) was used.

Buccal cell sampling

The technique used was adapted from the method by Liu et al. (16). In brief, the mouth was rinsed with clean water to remove saliva. The insides of the cheeks were brushed 3-5 times, with a clean, individually wrapped toothbrush, without causing any bleeding. Then the mouth was rinsed with 20 ml of isotonic saline solution, which was spit back into a 50 ml centrifuge tube. To recover buccal cells from the tooth brush, the brush was briefly agitated in the centrifuge tube.

Assessment of plasma zinc

Plasma samples were diluted twenty-fold with a 0.1% HNO_3 (GFS Chemicals, Powell, OH) containing 0.01% Triton X (octyl phenoxy polyethoxyethanol, T-6878 Sigma-Aldrich, Inc., St. Luis, MO). Zinc concentrations were assessed by inductively coupled plasma mass spectrometry (ICP-MS) (Perkin Elmer SCIEX Elan 9000, Perkin Elmer Life and Analytical Sciences, Norwalk, CT) using gallium as internal standard (Gallium 2%, Perkin Elmer Life and Analytical Sciences, Shelton, CT) and standard serum (Utak Laboratories Inc., Valencia, CA) as a quality control measure.

RNA extraction from RNAlater treated buccal cells

In the laboratory at Oklahoma State University, the buccal cells samples in RNAlater solution were centrifuged at 350 x g for 15 minutes. The RNAlater supernatant was stored separately. All buccal cell samples were extracted with TRIzol Reagent (Invitrogen, Ambion, Inc, Austin TX) according to the provided protocol. The RNA yield was assessed with the use of a UV spectrophotometer (Nano Drop ND-1000, Thermo Fisher Scientific Inc., Waltham, MA) at A260 and A280.

In addition, the RNA quality was also assessed through electrophoresis with Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA). A sample of 1µg of RNA was used to generate a double stranded cDNA with Super Script III Reverse-Transcriptase (Life Technologies, Carlsbad, CA) in a thermocycler (Thermocycler Biometra TGradient, Biometra GmbH, Goettingen, Germany).

The zinc transporter Zip4 and the MT mRNA expression of the samples were detected at baseline and at endpoint of the study by quantitative Real-Time PCR (7900

HT Fast Real-Time PCR System, Applied Biosystems, Forster City, CA). Therefore the 25 nmole DNA Oligonucleotide primers (19 bases) (Integrated DNA Technologies, Inc., Coralville, IA) were used (Table 1). The data were obtained as averaged triplicates, then ΔCt values (Ct target - Ct 18S) were assessed, using β-actin as housekeeping gene. The maximum ΔCt was subtracted from all ΔCt values, resulting in ΔΔCt values. Fold change in expression was determined by using the formula 2^-ΔΔCt. Primers are listed in Table 1.

Ethical approval

The small preliminary trial with ten volunteers was approved by the OSU Institutional Review Board. The goal of this trial was to develop a method of assessing buccal cell Zip4 mRNA and to establish a correlation between plasma zinc concentration and buccal Zip4 mRNA. The participants were students and staff at OSU.

The study with the Ethiopian volunteers was approved by the ethical committees of Oklahoma State University and Hawassa University. Furthermore, approval was granted on a regional level by the South Nations Nationalities and People's State Health Bureau, and by the Health Office of Awassa. On the national level, approval was granted by the National Health Research Ethics Review Committee of the Ethiopian Science and Technology Commission, and by the Drug Administration and Control Authority.

Results

This research project was performed in several steps.

Method validation: Identifying a simple method of buccal cell sampling.

The method by Liu et al. (16) was adapted for use in the preliminary US trials and in the Ethiopian field, as well as for several tests performed in the laboratory. A protocol with pictures was developed in order to support the volunteers and researchers in obtaining the buccal cells. The cells obtained with this method were successfully used for further assessment steps in several laboratory tests, before the method was used in the two trials.

Method validation: Identifying hZip4 and hMT1 in buccal cells.

Researchers assumed that hZip4 and hMT1 were expressed in buccal cells, but had to validate this assumption. Therefore, fresh buccal cell mRNA was extracted, and cDNA was synthesized followed by q-PCR of gene expression (Figures 1 and 2).

Method validation: Establish an association between dietary intake and mRNA of hZip4 and hMT1.

The correlation between dietary intake and expression of hZip4 and hMT1 was examined. Therefore the primary investigator (female, vegetarian for 28 years) obtained baseline buccal cells, then took zinc supplements in the form of zinc sulfate (50 mg zinc/day) for three weeks and again obtained buccal cells at the endpoint of the test phase. The cells were obtained twice on the same day, once in the morning and once in the late afternoon. The results shown in Figure 3 indicate that zinc supplementation decreased hZip4 RNA expression significantly. However, hMT1 RNA expression, which was expected to have a positive correlation to dietary intake, was also significantly decreased.

Preliminary trial in Stillwater: Correlation between plasma zinc, hZip4 and hMT1mRNA

In a smaller trial, blood and buccal cells from a group of 10 volunteers were obtained in order to determine if there was a correlation between plasma zinc and buccal cell hZip4 and hMT1 mRNA expression. No significant correlation between hZip4 mRNA (mean 24.9 ± 10.9), hMT1 mRNA levels (23.6 ± 12.8) and plasma zinc (82.2 ± 10.0 µg/dl) could be established (Table 2).

Assess quality of samples stored in RNAlater solution

Based on the conditions in the field, the researchers decided to use RNAlater in order to stabilize the samples during transport. The method was unable to be tested prior to the sample collection. To quantify the effect of RNAlater solution on the integrity of the sample quality, a small laboratory-based test was performed. Blood and buccal cell samples were obtained at baseline 1 and 23 days later at baseline 2. After collecting the data at baseline 2, the test person (female, vegetarian for 28 years) took a zinc sulfate supplement (50 mg zinc) for 20 days and again obtained blood and buccal cell samples.

To compare sample treatments, cooled, frozen, and RNAlater treated samples were obtained at three time points (Table 3). In order to mimic the conditions in the field, cooled samples were stored at 4°C for 3 days and then extracted and stored at -80°C. Frozen samples were frozen immediately after harvest at -80°C. Samples in RNAlater were stored at room temperature for 2 to 3 days and then at 4°C. To check for consistency, two baseline samples were taken 23 days apart from each other.

Table 3 indicates that RNAlater samples had larger pellets than most other samples, but the RNA yield was smaller and the 260/280 ratio of absorbance deviated further from the ideal 1.80 for RNA in the cooled and frozen samples.

The plasma obtained during the trial was immediately separated from the packed erythrocytes and analyzed in the ICP-MS (Table 4). Despite many years of ovo-lacto vegetarianism, all samples were well above 700 µg/L, the cut off for zinc deficiency. The baseline 2 sample deviated from the other two samples, with a very high zinc concentration.

In addition to the nanodrop assessment, the samples were assessed in the Agilent Bioanalyzer 2100 to test the quality of the extracted mRNA in the electrophoresis. Figure 4 indicates what intact RNA would look like in the electrophoresis printout (17). The samples in Figure 5 clearly show that the cooling (first row) resulted in a complete elimination of mRNA, probably due to protease activity. In the immediately frozen samples, only mRNA fragments could be identified. While in the mRNA samples stored in RNAlater, no 18 and 28s peak is visible at all. RNAlater seems to have destroyed the RNA in the samples almost completely or bound it too strong, so that it could not be extracted. The time span between harvest and extraction (at baseline 1, baseline 2, or endpoint) and therefore the difference in the length of storage did not matter for the quality of the RNA (Figure 5).

From the leucocytes, no cooled samples were produced (Figure 6). Frozen leukocytes did not show RNA protein peaks (18s and 28s) after the electrophoresis procedure. The comparison of leucocytes prepared along with the buccal cells shows that while there are some protein peaks in the RNAlater samples, the RNAlater sample with the storage duration of around 45 days did not have any visible proteins.

Whole blood samples (Figure 7) had some RNA proteins in the cooled and frozen samples. The cooled samples had only one of the two RNA peaks, the 28s peak at all time points. The frozen samples have the 18s and the 28s peak in all samples and at all time points. The RNAlater treated samples contain no RNA proteins based on electrophoresis analysis at any time point.

Discussion

The developed method using single wrapped toothbrushes and 50 ml centrifuge tubes with 20 ml saline solution is a simple method for use in the field. Furthermore, the data show that with this method, enough buccal cells could probably be obtained for use with the PCR technique. However, protease activity is possible; therefore precautions had to be taken. The samples of the small 10 volunteer trail at OSU were immediately processed, and in the Ethiopian trial RNAlater solution was used.

Furthermore, the data (Figure 1, 2) indicate that hZip4 and hMT1 can be identified in buccal cells in abundant amounts for scientific research. So far, hZip4 proteins were found in tissues primarily involved with the uptake of zinc, such as in the small intestine, colon, and kidneys (18). To our knowledge, hZip4 was not yet identified

in buccal cells. The same is true for hMT; although it is known to be in most cells, until recently it was not identified in buccal cells. hMT acts mainly as a detoxifier but has some antioxidant effect through its ability to release zinc (19). Recent research presented at the Experimental Biology 2011 meeting indicated a reduction in the MT transcription level in buccal cells with the qPCR method (20). This finding indicates that dietary depletion of zinc results in decreased MT mRNA expression, while in our research the dietary up-regulation through zinc supplementation did not occur (Figure 3). The small laboratory test showed that while hZip4 mRNA decreased during zinc supplementation, so did the hMT1. Despite the successful recovery of hZip4 in buccal cells with our method, buccal cells are not the primary site of zinc uptake and therefore the dietary dependence of the transport protein had to be demonstrated. Our data on hZip4 supports findings in the literature, where increased zinc availability led to significantly decreased hZip4 expression in human volunteers and cell cultures (11). In another study, zinc intake of only 15 mg for 10 days quickly (within a day) and significantly increased hMT mRNA expression, assessed in qRT-PCR technique in leukocytes and whole blood (dried blood spots). However the authors did not report plasma or serum zinc levels at the beginning and end of the supplementation trial (13).

In the primary trial at OSU, blood and buccal cell samples of 10 volunteers were obtained. The goal was to investigate the correlation between plasma zinc and mRNA expression of hZip4 and hMT1 (Table 2). The correlations were extremely weak and none of them were significant. However, studies in human intestinal cells show that there is a correlation between low zinc intake and high mRNA expression for zinc transporter hZip4 (7, 21). Studies in animal models and humans support this direct connection

between hZip4 mRNA and dietary zinc intake. Researchers either find an up-regulation of hZip4 mRNA due to decrease of availability, or a decrease of hZip4 mRNA due to increased zinc availability (10, 11). Thus, the dietary dependency of hZip4 makes this transporter an ideal biomarker for zinc intake. However, we did not find any source in the literature where the direct correlation between blood zinc levels and mRNA expression could be established. The reason is that plasma or serum zinc may not reflect true zinc status, and therefore the measured zinc in this compartment can be seen more on a population level, rather than on an individual level (6). Furthermore, Zip4 mRNA expression is significantly correlated to zinc availability in Caco2 cells, a mucosal cell line (22). The development of a non-invasive method to determine zinc status, for example in buccal cells, would potentially enhance knowledge about zinc and increase the understanding of nutrition in the field, not only in the developing world but also globally (23).

Despite the fact that many researchers show similar to improved recovery of mRNA from both cell and tissue samples stored in RNAlater solution, as compared to frozen samples (24) (25, 26), we could not recover sufficient mRNA from our samples. Even mRNA from samples stored in RNAlater for a short time did not contain enough detectable mRNA for the downstream processes (Figures 5-7). The assessment of the quality of the RNA with the electrophoresis technique revealed that the leukocytes stored in RNAlater (Baseline 2 and Endpoint) and frozen whole blood samples (Whole Blood -80ºC) might be used for the qPCR. The quality of the RNA obtained in the electrophoresis was not high, despite the good yield and the 260/280 ratio close to target (Figure 5, Table 3).

In conclusion, with the developed method we were able to gain sufficient amounts of RNA for the qPCR method. Furthermore, we demonstrated that hZip4 and hMT1 can be found in buccal cells; while we were able to show the dietary dependency for hZip4, we could not establish the same for hMT1. We were also not able to show a correlation between hZip4 mRNA expression and plasma zinc concentration. Due to the fact that we used RNAlater for our samples in Ethiopia, we did not succeed in extracting RNA from the samples with our method. However, we gained high quality RNA from small amounts of leucocytes, and the extraction of cooled and frozen buccal cells yielded somewhat usable RNA, while the RNAlater stored samples of both contained no RNA.

This research was supported by the Swiss National Science Foundation (PBSKP3-124358), NIH Grant R01HD053053 (NICHD & Fogarty International Center), Hawassa University, and Oklahoma State University. We thank Meron Girma and her team at Hawassa University for their technical support.

Table 1: List of the primers for Zip4, MT1 and the housekeeping gene β-actin with accession number, sense and antisense primers used for this study.

Gene	Accession number	Sense Primer	Antisense Primer
Zip4/SLC 39A4	NM_130849.2	5’ AAT AGG GAC GAT TGC CTG AT 3’	5’ GCC AGG CCA TCG AAA T 3’
MT1	NM_005946.2	5’ CTC CTG CTG CCC CAT GAG 3’	5’ TCT GAT GCC CCT TTG CAG 3’
β Actin	NM_001017992.2	5’ GCA CCC AGC ACA ATG AAG AT 3’	5’ GCC GAT CCA CAC GGA GTA C 3’

Table 2: Pearson's correlation coefficients and p-values of hZip mRNA expression (1), hMT1 mRNA expression (2), and plasma zinc (3).

		1	2	3
1	hZip4 mRNA	1	0.354; $p = 0.436$	0.036; $p = 0.945$
2	hMT1 mRNA		1	0.091; $p = 0.846$
3	Plasma zinc			1

Table 3: Test of the effect of storage conditions on buccal cell samples, with comparison of pellet size (non visible, small, large, and very large), RNA yield in ng/, and 260/280 ratio of absorbance for RNA. The storage conditions were firstly cooling at 4°C for 3 days, secondly immediate storage at -80°C and thirdly treatment with RNAlater followed by storage at room temperature for 2 days and 4 °C starting from day 4, while the first samples were stored at -80°C after 3 weeks. [1]

		Baseline 1			Baseline 2			Endpoint		
Treat-ment	Stor-age	Pellet size	RNA (ng/µL)	260/ 280	Pellet size	RNA (ng/µL)	260/ 280	Pellet size	RNA (ng/µL)	260/ 280
Buccal cells cooled	4°C 3 d	No samples			**	879.6	1.84	-	583.7	1.71
Buccal cells frozen	-80°C	**	2801.1	1.94	*	920.1	1.79	-	560.3	1.58
Buccal cells in RNA-later	RT 2-3 d, then 4°C	**	106.7	1.65	***	122.5	1.48	**	52.1	1.48

[1]Pellet size: - non visible pellet; *small pellet; **large pellet; ***very large pellet;

Table 4: Results of plasma zinc analysis of the baseline 1 and 2 as well as the endpoint sample in ICP-MS. The Plasma samples were obtained with trace mineral free equipment. Sample Baseline 2 had a slight hemolysis and therefore an elevated zinc concentration.

	Baseline 1	Baseline 2	Endpoint
Plasma zinc (µg/L)	818.0	1532.2 (slight hemolysis)	931.4

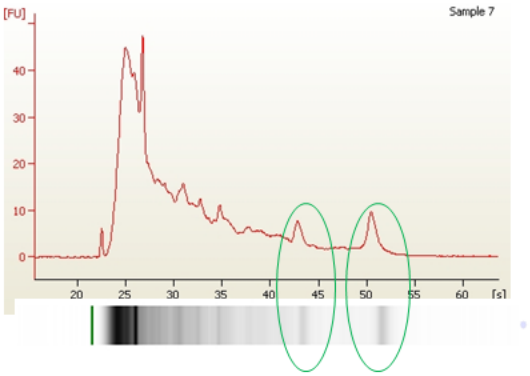

Figure 7: Example mRNA extracted from buccal cells. Electrophoresis of a sample (sample 7) of fresh buccal cell RNA in the Agilent Bioanalyzer 2100. RNA is visible as two peaks, one after 39 seconds (18s) and one at 46 seconds (28s) (compare Figure 4).

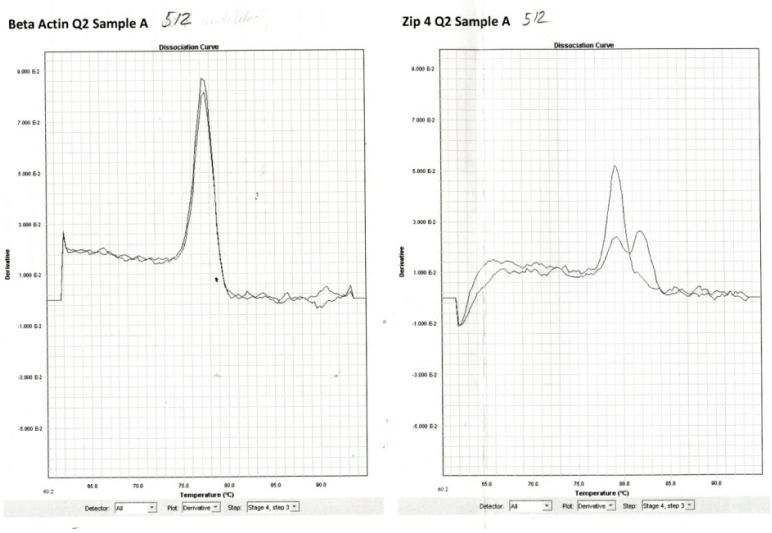

Figure 8: Dissociation curve of one buccal cell sample from the US trial (code 512). On the right is the housekeeping gene β-actin amplicon, and on the left is the Zip4 amplicon. Both samples were performed in duplicates, visible as two lines.

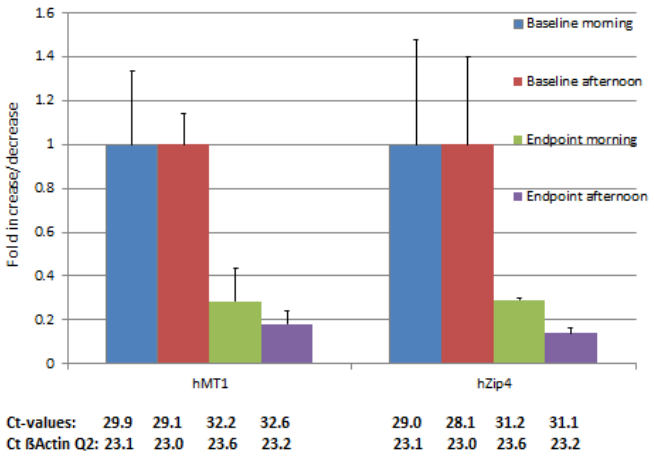

Figure 9: Human MT1 and Zip4 mRNA expression, before and after the dietary supplementation of one vegetarian with 50 mg zinc daily for 3 weeks. The data were normalized based on baseline morning and afternoon samples expressed as fold increases with standard deviations.

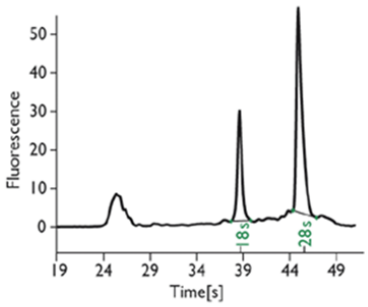

Figure 10: Example of electrophoresis profile (Agilent Bioanalyzer 2100), of RNA, 18s visible after 39 and 28s RNA after 46 seconds respectively (Source: Invitrogen/Ambion).

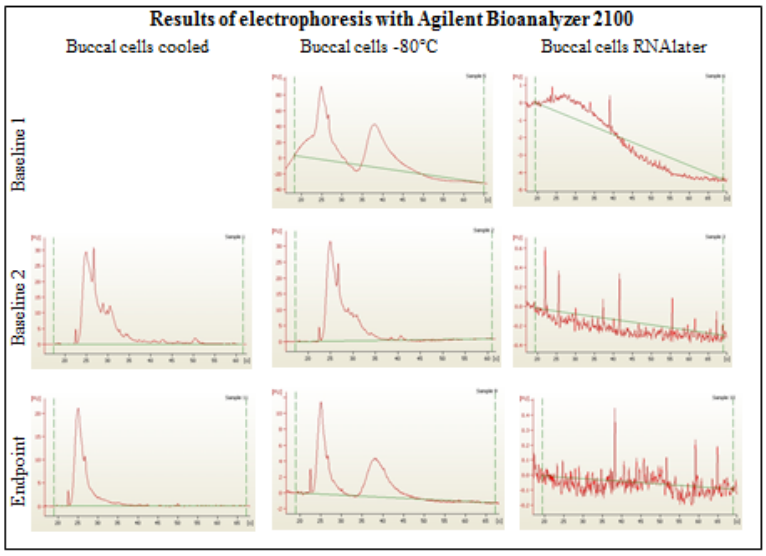

Figure 11: Electrophoresis charts of buccal cells cooled, frozen, and stored in RNAlater solution at baseline 1 and 2, as well as at endpoint.

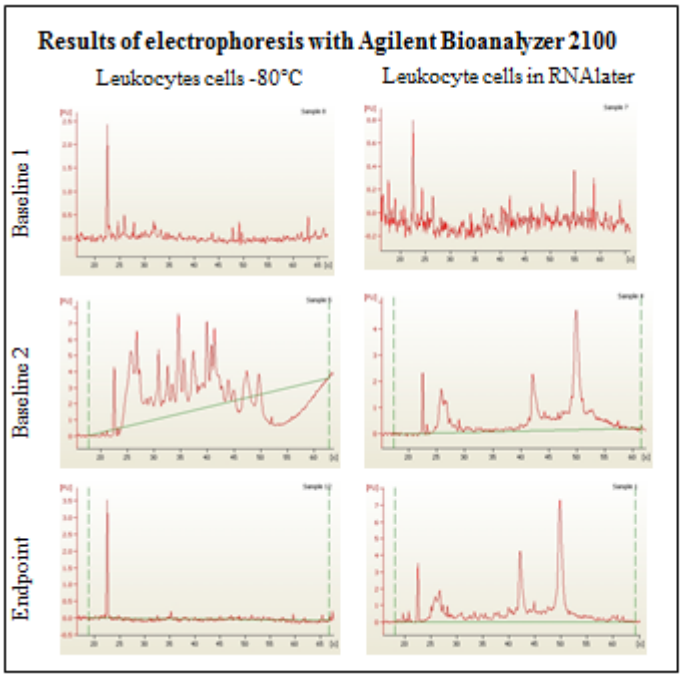

Figure 12: Electrophoresis charts of leukocytes stored at -80°C and in RNAlater at the three time points (baseline1, baseline 2, and endpoint).

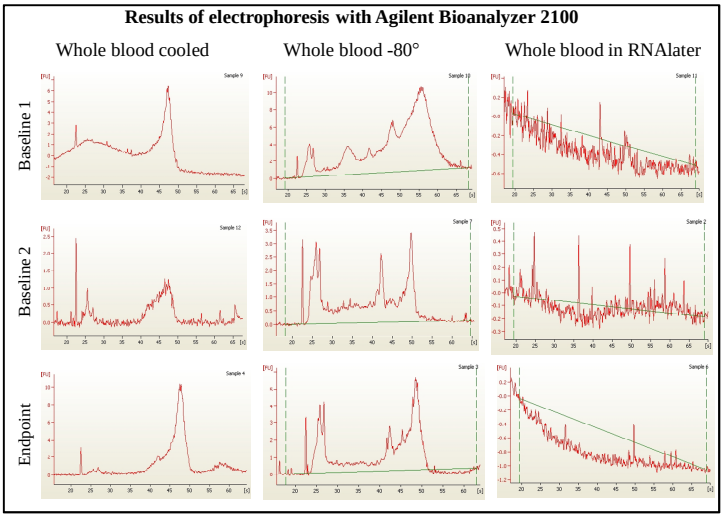

Figure 13: Electrophoresis chart of whole blood cooled, at -80°C and in RNAlater at the three time points (baseline1, baseline 2 and endpoint).

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CHAPTER V

CORTISOL LEVELS MEASURED IN DIFFERENT COMPARTMENTS IN WOMEN FROM SIDAMA, ETHIOPIA

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Abstract

Cortisol is a corticosteroid hormone whose release is triggered by stress, anxiety, and inflammatory cytokines, making it an important stress marker. Techniques for measuring cortisol have changed significantly over time, and at present, enzyme-linked immunosorbent assay (ELISA) methods are often employed in clinical settings.

This study measured saliva, serum, and urine cortisol in rural Ethiopian women. The comparison of the cortisol concentrations in different compartments revealed that urine and saliva cortisol were correlated to each other ($r = 0.36$, $p < 0.05$), but not to serum cortisol. Furthermore, saliva cortisol concentrations in 25% of the volunteers were marginally above the reference value of the ELISA kit used, compared to 7.5% above

reference in serum. All urinary cortisol concentrations were within the reference values. There was no correlation between any cortisol measurement and high sensitivity C-reactive protein (CRP) or serum albumin. Urinary cortisol was correlated with urinary total protein ($r = 0.41$, $p < 0.01$) and urinary iodine ($r = 0.50$, $p < 0.01$). In addition, serum cortisol was negatively correlated to total antioxidant capacity ($r = -0.50$, $p < 0.01$) measured by the ferric reducing antioxidant power (FRAP) assay. CRP was negatively related to serum albumin ($r = -0.43$, $p < 0.01$).

Our study results indicate that despite iodine deficiency cortisol concentrations were within normal ranges or marginally higher. There was also little correlation between different health markers in this sample of moderately iodine deficient women.

Introduction

Cortisol is a glucocorticoid hormone from the hypothalamic-pituitary-adrenal (HPA) axis that acts as an important stress marker (1, 2). Ever since the first studies of cortisol by Selye (3), stress has been considered a trigger for increased circulating cortisol levels. In general, increased cortisol is triggered by inflammatory cytokines in order to exercise the anti-inflammatory effect of cortisol (4). In addition to its anti-inflammatory properties, cortisol clearly has an effect on the immunological function of the body (5). Elevated cortisol concentrations thereby were found to be involved in increased cell death, mainly in cells without the antiapoptotic Bcl-2 protein (6) (7). Among the non-Bcl-2 containing cells are immature B and T-cells. A decreased number of immature T-cells eventually leads to thymus atrophy (6, 8).

In addition to immunological constraints, protein energy malnutriton is often associated with increased serum cortisol levels (9-11). For example, elevated cortisol concentrations in urine, serum, and saliva have been demonstrated in 47 female anorexia nervosa patients compared to a healthy control group (12). Mean cortisol levels were also found to be well above normal, and significantly higher than those of a well-fed control group, in children with kwashiorkor and marasmus (13). As kwashiorkor developed, cortisol levels increased in conjunction with a decrease in serum albumin concentrations. However, serum cortisol stayed normal until the albumin level was below 30 g/L. According to the researchers, serum hypoalbuminemia was correlated to infection. Thus, cortisol increase was most likely due to infection (14).

To test whether cortisol could be associated with malnutrition, we obtained saliva, serum, and urine samples from women of the Sidama region of Ethiopia, one of that nation's most food insecure regions (15). In addition to food insecurity, iodine deficiency is a common problem in this region. For example, one study found that mean urinary iodine concentrations in pregnant Sidaman women were 58.0 µg/L, well below the recommended 100-199 µg/L for adult women (16, 17). In addition 40.2% palpable goiters as well as 16.0% visible goiters were observed in Sidaman children aged 6-12 (17). To our knowledge, no recent studies of this population have addressed the relationship between cortisol and iodine deficiency caused by low iodine uptake. Research on hypothyroidism in patients with Hashimoto’s thyroiditis indicated no significant elevation in urinary free cortisol, however half of the patients were taking prescription medicine, which might have reduced mean cortisol concentrations (18).

Elevated cortisol concentrations suppress the thyroid releasing hormone production in the hypothalamus and in addition, elevated inflammatory cytokines may inhibit transcription of enzymes which activate thyroid hormones (19, 20). In turn, hypothyroidism results in somewhat decreased cortisol concentrations (21). These findings are the base for the reciprocal theory (Figure 1) (19). However, there is rising evidence that long term stress due to chronic illness, or in the aftermath of traumatic events such as in post-traumatic stress disorder, can lead to hypocortisolism (22). The exact mechanism of how long term stress causes hypocortisolism is not yet clear, although exhaustion of HPA axis production and changes in HPA axis function may be possibilities (22).

Cortisol can be measured in three main compartments: urine, serum and saliva. Saliva tests were investigated in this study because they are non-invasive and easy to administer in the field in Africa. Saliva is composed of the fluids from three main glands in the oral cavity, as well as exudates of orophagal mucosal cells that produce crevicular fluids. Remarkably, cortisol concentrations do not vary with salivary flow (23). Although urinary cortisol tests in 24 h urine were once the preferred method, salivary tests are now more frequently employed. For the crushing syndrome screening for example, 24 h urinary or salivary cortisol are recommended as being equally useful (24). Comparing cortisol measurements from different compartments is difficult, because of confounding factors (25-27). Urinary cortisol for example, is influenced by liquid intake (28).

In addition to the difficulties when comparing different compartmental measurements, the comparison between different laboratory methods is also problematic. Methods such as radioimmunoassay (RIA), ELISA, and high performance liquid

chromatography (HPLC) yield widely differing normal ranges when used to test healthy subjects (29). For example, RIA test kits often show cross-reactions between urinary cortisol and other substances, but ELISA kits do not (25, 29). However, when urine was pretreated prior to analysis in RIA and ELISA test kits, a strong, significant correlation between the results (r = 0.93) (30) was found. In the case of plasma cortisol, pretreatment of plasma is not necessary to reach equally strong correlations within the RIA and ELISA methods (31). Because of the difficulty in comparing results obtained by different test kits, we used one ELISA kit to test all samples, using the given reference ranges to compare results, as suggested by Gatti et al. (25).

Because cortisol is strongly related to infection and inflammation, several additional health markers were assessed, including high sensitivity C-reactive protein (CRP), α-1 acid glycoprotein (AGP), serum albumin, and urinary total protein and urinary iodine (32, 33). Furthermore, ferric reducing antioxidant power (FRAP) was measured in order to assess antioxidant capacity and identify reduced levels possibly caused by increased oxidative stress (34, 35).

This study had three main goals. Firstly, to compare serum, urinary and saliva cortisol of forty Ethiopian women to evaluate the validity and feasibility of collecting cortisol samples from saliva in the field in Africa. Secondly, the cortisol data was correlated to different infection and inflammation markers in the markedly iodine deficient sample. Thirdly, the different health markers were correlated to each other.

Volunteers, materials and methods

Volunteers, study design, supplementation and sample collection

Forty non-pregnant women, age 20 to 45 years, from the rural Finichawa village in the Sidama region of Ethiopia provided oral consent to participate in a placebo-controlled zinc supplementation trial. All data was collected in January 2010. A local health worker excluded volunteers with obvious signs of inflammation or impaired health.

Saliva, serum, and urine samples were collected in the morning from the fasting volunteers. Saliva was collected with salivette polyester pads (Polyester Salivette, Sarstedt, Newton, NC) which were placed in the mouth of the volunteers, who chewed on them. Once soaked with saliva, the pads were placed back in their shell. Tubes containing the pads were then sent to the Hawassa University laboratory, where they were centrifuged at 350 x g. The serum was collected with sterile equipment suitable for trace element analysis (Sarstedt, Newton, NC) by venipuncture. The volunteers also collected a random urine sample in a cup. All samples were kept on ice after collection and at 4° C in the laboratory of Hawassa University; they were shipped on dry ice from Ethiopia to the U.S. for further analyses.

Cortisol in urine, serum, and saliva

Cortisol was measured with commercial 96-well ELISA plates (Parameter Cortisol KGE008, R&D Systems, Minneapolis, MN), and the analysis was performed with a plate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA). Quality controls (Control Set #568 RD6, R&D Systems, Minneapolis, MN) were used with low

(0.68-1.33 ng/mL), middle (1.99-3.76 ng/mL), and high (3.74-5.95 ng/mL) cortisol samples. Urinary cortisol is often measured in urine collected over 24 h, and free serum cortisol is frequently calculated from the results of a total cortisol and a cortisol binding protein kit (25, 36). Normal total serum cortisol in the literature ranges from 72.46 to 289.86 ng/mL (200 to 800 nmol/L) (37). In our ELISA test kit the total serum cortisol in a sample of healthy adults was 16.8 to 75.5 ng/mL (46.37 to 208.39 nmol/L). However, the kit has a low recovery rate for serum cortisol with a range of 30-55%. Because no single method of cortisol analysis has been established to be the most effective, we used one kit only for all analysis and used the reference ranges for every compartment given by the manufacturer (1, 26). The manufacturer's reference range for saliva was 0.8 to 2.9 ng/mL, well within the 0.94 to 15.14 mg/mL (2.6 to 41.8 nmol/L) range found in healthy women aged 31 to 40 years (37). The manufacturer's reference range for urinary cortisol was 20.8 to 153.0 ng/mL (57.41to 422.31 mmol/L).

Iodine in urine

Urinary iodine was measured with a modified protocol of the Sandell-Kolthoff reaction method (38), using ammonium persulfate for the digestion of the urine (39). Absorbance at 405 nm was measured using a SU 800 spectrophotometer (Beckmann Coulter, Fullerton, CA). Iodine standards from 0-300 µg/mL were used to linearly interpolate the obtained absorption and to calculate iodine absorption (GraphPad Software, Inc., La Jolla, CA).

Urinary iodine levels provide an accurate assessment of community levels of deficiency, although not the iodine status of a single person. Volunteers were grouped into

the four different classes of iodine status defined by the World Health Organization: severe iodine deficiency (< 20 µg/L), moderate deficiency (20-49 µg/L), mild iodine deficiency (50-99 µg/L), and adequate iodine nutrition (100-199 µg/L)(40).

High-sensitivity C - reactive protein (CRP) in serum

CRP is produced in the liver in acute conditions of trauma, infection or inflammation. CRP levels were analyzed using a sandwich ELISA test kit (961CRP01H, Helica Biosystems, Inc., Fullerton, CA). Optical density was assessed at a wavelength of 450 nm in the plate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT). The linear standard curve created was used to interpolate the sample and control concentrations. The controls (Kentrol Reference Serum) were within the expected ranges. The kit manufacturers reported serum CRP concentrations of 3.8 ng/mL and below (n = 59) measured in healthy individuals. The cutoff for elevated CRP was assumed to be ≥ 3 mg/L for this study (41).

α -1-acid glycoprotein (AGP) in serum

The hepatic acute phase protein AGP was assessed using a sandwich ELISA quantification kit (40-288-22927F, GenWay Biotech, Inc., San Diego, CA). The calibrators, serum (dilution 1:8503) and control (Kentrol Reference Serum) were added to the coated wells. The 96-well plate was then read at 450 nm in the plate reader. The standard curve was calculated with a four parameter logistic curve fit, and the AGP concentrations in serum and controls were interpolated based on the standard curve. The

controls lay within the expected range. AGP levels above 1.2 mg/L indicate inflammatory processes.

Albumin in serum

Serum albumin was assessed with a reagent kit (BQ 002 CR, BioQuant Diagnostic, San Diego, CA). Absorbance at 630 nm is proportional to the albumin concentration. The standards from 0 to 7 g/dL were prepared; controls (GEMCAL Reference Serum, Alpha Wassermann Diagnostic Technologies, LLC, West Caldwell, NJ) and samples were diluted twofold for the analysis. The samples were then measured in the plate reader. Albumin concentrations of the controls were within the reference level. Albumin concentrations within 3.5 and 5.3 g/dL were considered normal (42).

Total protein in urine

Urinary total protein was assessed using the bicinchoninic acid (BCA) test kit (PI 23227, Thermo Fisher Scientific Inc., Hanover Park IL). Urine samples and controls (GEMCAL Reference Serum) were diluted tenfold and pipetted into 96-well plates. Absorbance at 562 nm was measured after incubation for 30 minutes in the plate reader. Controls were within the expected range. Working range of the method when using a 25 µL sample is 20-2000 µg/mL; normal range for urinary protein is 10-80 mg/L in healthy adults.

Ferric reducing antioxidant power (FRAP) in serum

The FRAP assay was based on the method developed by Benzie and Strain in 1996 (43). For the FRAP measurement, a 10:1:1 mixture of acetate buffer pH 6 (300 mM, sodium acetate trihydrate S-9513, Sigma-Aldrich, Inc., St. Louis, MO, dissolved in glacial acid, 281000ACS, Pharmco-AAPER, Brookfield, CT), 2,4,6-tripyridyl-s-triazine (10 mM TPTZ, 70204-1EA-R , Sigma-Aldrich, Inc., St. Louis, MO, dissolved in 40 mM HCl, H1035, Spectrum Chemical Mfg. Corp. New Brunswick, NJ) and iron chloride (20 mM $FeCl_3$*6 H_2O, 1-1996, J. T. Baker, Phillipsburg, NJ) was produced. Iron sulfate ($FeSO_4$, FX265, MBC Manufacturing Chemists, Inc., Cincinnati, OH) served as standard.

One hundred and fifty µL of the working reagent was pipetted into 96-well plates, and the absorption at 593 nm was measured in the plate reader. Twenty µL of standard or serum was added to the wells. After shaking the plate well, it was covered and stored at 37°C for 8 minutes. The optical density was again measured at 593 nm, and the difference between both absorptions was calculated. A linear calibration curve was drawn from the standards, which served as the basis to interpolate the serum concentrations. Healthy adults have FRAP values between 600 to 1600 µmol/L (43).

Statistical analysis

Pearson's correlation coefficients were calculated using the statistic software SPSS/PASW 18.0 (IBM Corporation, Somers, NY). The data were expressed as mean and standard deviation (SD), and the interquartile range of 25% and 75% was assessed.

Ethical approval

Approval for the study was given by the ethical committees of Oklahoma State University and Hawassa University. Approval was also granted through two regional government agencies: the Southern Nations Nationalities and People's State Health Bureau, and the Health Office of Awassa. At the national level, approval was granted by the National Health Research Ethics Review Committee of the Ethiopian Science and Technology Commission, and the Drug Administration and Control Authority.

Results

The mean cortisol levels were within the normal range for the ELISA kit for all of the urine samples and most of the serum samples (Table 1). However, one quarter of the saliva samples were outside the given range for healthy adults. The interquartile range (median and the 25% and 75% percentiles) for the samples shows that most measurements were within the range given by the ELISA kit and all are within the reference range for healthy adult women aged 30 to 41 found in the literature (37). Some of the serum samples were above the R&D Systems kit reference levels but still well within the reference given in another study, which reached up to 289.86 ng/mL (37).

Data analysis revealed only one correlation, a weak significant linear correlation between urine and saliva cortisol concentrations (Table 2). A relatively weak and non-significant negative correlation of urine and serum cortisol was found (r = -0.307, p = 0.054).

The mean and standard deviation of urinary iodine concentration was 32.3 ± 31.2 µg/L, and a median and standard deviation of 22.7 ± 31.2 µg/L. Only eight out of forty volunteers had urinary iodine concentrations above 50 µg/L, and these ranged from 52.0

to 171.2 µg/L. Eleven of the volunteers had elevated CRP (>3 mg/L) concentrations (mean 5.8 ± 2.0) and the total sample had a mean, and standard deviation of 2.3 ± 2.5 µg/L , and a median of 1.1 ± 2.5 µg/L. All AGP levels were in the normal range (<1.2 g/L) showing a mean and standard deviation of 0.3 ± 0.1 g/L. Mean serum albumin concentrations were 4.3 ± 0.3 g/dL, and all were within the normal range . All urinary total protein levels were within the normal range, at 5.9 ± 1.7 mg/L. The FRAP assay results indicated that 49.4% of the women had serum levels below the normal range of 600-1600 µmol/L with a mean of 554 ± 208 µmol/L. In summary, CRP concentrations in some women were above the recommended range, while AGP, serum albumin, and urinary total protein were within the normal range. All of the FRAP measurements suggested low levels of antioxidant capacity.

Urinary cortisol was correlated to urinary iodine, while serum cortisol was positively correlated to AGP and negatively to FRAP (Table 3). Furthermore, saliva cortisol was significantly positively correlated to FRAP. The Pearson's correlations coefficients in Table 4 show only one significant correlation between the surveyed parameters, a significant negative correlation between CRP and serum albumin. AGP was not significantly correlated any other acute phase protein or urinary iodine.

Discussion

Cortisol concentrations in the urine and serum of these moderately iodine deficient women were normal. A quarter of the saliva measurements were above the reference range of the test kit (Table 1), although these still fell within the normal range of healthy women found in another study (37). Although blood contamination could be

responsible for the elevated cortisol in saliva, no injury to the buccal mucosa was likely due to the polyester pads used for the saliva collection and none of the samples were pink (23).

One possible explanation for the higher cortisol concentrations in the saliva is that they resulted from study participants' anxiety in the unfamiliar setting (a "white coat" effect), as it took several hours to collect the samples and fill out questionnaires. This assumption is supported by previous studies which found that cortisol levels rise rapidly under stress (1, 26, 27). However, because all samples were taken after at least an hour of screening and interviews, a "white coat" effect should have also affected serum and urinary samples, not just the saliva samples. The "white coat" effect is of particular concern to researchers doing fieldwork in rural Africa and should be subject to further investigation.

It is generally difficult to compare cortisol measurements in different compartments, because they measure different aspects of HPA axis activity, and because urinary cortisol measurements may be confounded by other substances (28, 44). Still, some researchers tend to use cortisol measurements in saliva, serum and 24 h urine interchangeably, a practice criticized by Yehuda et al. (27). In our sample we could not establish a correlation between serum cortisol and any other cortisol measurement, which is consistent with research on elevated cortisol concentrations (Table 1) (26, 46).

Some studies have found a high correlation between cortisol concentrations in serum and saliva, despite the fact that saliva cortisol is typically free cortisol, while serum cortisol is mainly bound to cortisol binding globulin (45). Poll et al. (44) reported a strong correlation between free saliva cortisol and calculated free cortisol measured in

serum (r = 0.836), after taking multiple measurements over two days. However, the morning free saliva cortisol and serum total cortisol measurements were not significantly correlated with each other (r = 0.404, p = 0.247) (44).

Further studies found no linear correlation in free serum and saliva cortisol of three groups of women, women with and without contraceptive use as well as in pregnant women. Contraceptive use and pregnancy increased cortisol binding globulin, which in turn bound more cortisol in plasma. In these studies the free available cortisol in plasma was not correlated to saliva cortisol (26, 46). Another study found a positive (r = 0.86, p < 0.0001) relationship between single morning serum and saliva samples below 163.04 ng/mL (450 nmol/L) (37).

Salivary cortisol is considered more reliable than serum or plasma levels by some researchers, as the stress caused by blood drawing can induce cortisol production (23). Gozansky et al. found that free saliva cortisol measurements showed dynamic changes faster and more accurately than serum total cortisol (47). In their study, healthy volunteers were injected with cortisol releasing hormone and forced to exercise. The relative increase in saliva cortisol to both challenges combined was 697 ± 826% and in serum 290 ± 150% (p = 0.04). The overall sample (n = 183 pairs) had a significant correlation (r = 0.60, p < 0.001) between serum and saliva. Separating the data of women taking oral estrogens (n = 52 pairs, r = 0.75, p < 0.001), and of the group not taking estrogens (n = 131 pairs, r = 0.62, p < 0.001) strengthened the linearity of the correlation between serum and saliva measurements for both groups (47). Saliva cortisol was shown to be a reliable tool to assess cortisol for several distinct applications (48). In our sample saliva cortisol appeared to be elevated in a quarter of the sample based on the R&D

Systems reference ranges but were well within the normal range for healthy women aged 31 to 40 (37) (Table 1).

The 24 hour urine collection allows for the assessment of the complete circadian cycle; however other substances may interfere with the free cortisol measurement. Urinary cortisol measured in ELISA plates represents free urinary cortisol. In our sample we demonstrated a significant but weak correlation between urinary and saliva cortisol ($r = 0.36$, $p = 0.025$). Both of these measurements represent free cortisol concentrations of their respective compartments (Table 2) (1, 23). A study comparing urinary cortisol (in 24 hour collected urine) and saliva cortisol concentrations (measured six times during the same day) in healthy participants could not establish an association between the different measurements. However, the researchers found that the diurnal saliva sample, collected early in the morning, was a good indicator for overall cortisol production measured in the urine sample ($r = 0.32$, $p < 0.05$) (27). Therefore the saliva cortisol in our sample could be seen as a predictive measurement for the urinary cortisol concentrations over the whole day**,** and might be more reliable than the one time urinary measurement.

This study found a non-significant negative correlation ($r = -0.31$, $p = 0.054$) between serum cortisol and urinary cortisol. In the literature no explanation for this possible negative relationship could be found. Overall the cortisol concentrations in our sample were normal to slightly elevated, based on the ranges given by the test kit and in the literature (Table 2) (48).

The researchers in this study assumed that high cortisol concentrations would be found in this region of Ethiopia, because of the increased risk of infection and inflammation caused by malnutrition. One previous study had shown that the rural

Sidaman diet consists of very little dairy or milk, practically no meat, little fat**,** and limited varieties of vegetables. Nearly 85% of the energy came from two food items only, fermented enset and unleavened maize bread (50). Common nutritional deficiencies in this region include protein-energy malnutrition, and vitamin and mineral deficiencies (33, 50). However, data is not conclusive whether malnutrition without current inflammatory processes would lead to increased cortisol concentrations. One research study indicated that malnutrition alone did not increase cortisol concentrations, but that increased serum cortisol concentrations were found in malnourished children who also suffered from infections (14). Well-nourished children (mean age 36.7 months) with infections had even more elevated serum cortisol (median 24.4 µg/L) concentrations than the marasmic group (median 14.2 µg/L) with infections, although this difference was not significant. Inflammation markers such as IL-6 and TNFα were not increased in the marasmic only (median cortisol 5.1 µg/L) group, while the other groups showed increased levels (51). Also in a severely (mean cortisol 9.32 nmol/L) and moderately malnourished (mean cortisol 7.74 nmol/L) sample of children (12-71 months) from Brazil, saliva cortisol concentrations were within the normal range and not significantly different from each other (52). Despite these results in children, some studies show that malnutrition alone may lead to elevated cortisol concentrations, which was expected in our sample of forty women (5, 9). For example in a rat study food restriction (40% for 25 days) alone increased cortisol concentrations significantly (53). Another study where the animals were restrained in order to increase stress, cortisol concentrations increased significantly, without an increase in inflammatory markers (20). Our findings of urinary, saliva and serum cortisol are not supporting the results of these rat studies. Despite of the

monotonous diet, and moderate iodine deficiency, cortisol concentrations were at normal levels. This is consistent with a study in hypothyroid women (30-57 y) that had urinary cortisol concentrations within the normal range of 55-268 nmol/day measured in 24 h urine (18, 21).

Research in the Sidama region showed high goiter rates in adults (17). Mean urinary iodine in our sample of women living approximately 2000 meters above sea level was 32.3 µg/L, indicating a moderate iodine deficiency. The low urinary iodine in this group of women was probably due to an extremely low intake of iodized salt paired with a high intake of kale which contains goitrogens. The low iodine concentration in the sample was not due to losses while handling or transporting from the field in Ethiopia to the laboratory at Oklahoma State University, as iodine is relatively stable in urine samples and evaporation would have increased rather than decreased it the iodine content (16).

Research indicates that there is a possible connection between increased cortisol concentrations and decreased thyroid parameters. For example, the food restriction in the above mentioned rat study, did increase cortisol concentrations significantly, while triiodothyronine (T_3), thyroxine (T_4) and thyroid-stimulating hormone (TSH) were significantly reduced compared to the control (53). The second study in rats indicated that stress induced by restraining the animals, increased cortisol levels significantly. In addition, serum T_3 and 5'deiodoidase activity in liver and kidney decreased time-dependently. Despite significantly decreased TSH concentrations, T_4 did not decrease within the 8 hour test phase (20). These results support the reciprocal theory of an inverse relationship between TSH and cortisol concentration in serum (Figure 1). For our

research we did not measure TSH, T_3 and T_4, therefore we cannot determine if the low urinary iodine of our sample is only due to low intake of iodine, or at least partly due to the mechanisms of the reciprocal theory.

The correlations between cortisol measurements and inflammatory parameters as well as FRAP and urinary iodine revealed that urinary cortisol was significantly and positively correlated to urinary iodine and total protein measurements (Table 3). Based on the above mentioned literature, increased cortisol decreases TSH concentrations which are associated to increased urinary iodine excretion, when iodine intake is not limited (Figure 1) (55). Furthermore, proteinuria was connected to inflammation in otherwise healthy adults (19-63 y) from 1.29 g/24 h versus 0.09 g/24 h in an age-matched group (58). Increased inflammation in turn would increase cortisol levels (51). However, we could not establish the correlation between cortisol and CRP (study showed mean of 24.5 ± 2.3) concentrations ($p < 0.001$), as indicated in a study with 115 Crohn's disease patients 18 to 48 years of age. While serum cortisol was positively correlated to the inflammation marker AGP, it was negatively correlated to FRAP. Serum and saliva cortisol concentrations show contradictory correlations concerning FRAP measurements. In the literature contradictory results can be found as well, while in one study FRAP and cortisol significantly increased during the exercise regimes (59). Another study found significantly decreased antioxidant concentrations (measured as ORAC) in high cortisol concentrations due to nephrotic syndrome compared to a control group ($p = 0.011$) (60).

The correlations between the different health markers were also evaluated (Table 3). The serum CRP concentrations were increased in some volunteers (11 women) in our study, indicating acute inflammatory processes in some of the volunteers. A weak but

significant negative correlation was found between serum albumin and CRP. Despite the low iodine levels in our sample the common inflammation markers AGP and serum albumin were not elevated. None of the other measured parameters was significantly correlated to each other most likely because they do not directly depend on each other and have different underlying mechanisms. In the literature, urinary iodine concentrations below 100 µg/L in 56 children were not significantly correlated to AGP concentrations, while concentrations above 100 µg/L in 60 children were significantly correlated to AGP. In addition, all CRP measurements for both groups were within the normal range (61). In another study increased urinary iodine concentrations were associated with increased alterations in the glycosylation of acute phase proteins containing glucose molecules such as AGP. CRP was not elevated in thyroid disease, unless there was a simultaneous acute inflammatory process, such as in untreated sub-acute thyroiditis where CRP concentrations rose to a mean of 33.4 mg/L ($p < 0.00001$) (62).

In conclusion, free cortisol, assessed in urine and saliva were significantly correlated to each other, while serum cortisol was not correlated to the other measurements. Despite the moderately low urinary iodine concentration in our sample, as well as a monotonous diet, cortisol levels were within the normal ranges, except for the saliva measurements where one quarter of the samples had slightly elevated concentrations based on the test kit used. Based on concentrations found in the literature, all cortisol concentrations were in the normal range. Our study found only a few weak correlations between the different health markers.

The limitation of the study is due to the fact that the ELISA method is somewhat impaired in assessing the serum cortisol levels, indicated by the recovery range of 30-

55% from cortisol spiked samples. The study results would needed further confirmation with another assay method. Furthermore, in order to assess the reciprocal theory, we would need to assess several thyroid markers.

This research was supported by the Swiss National Science Foundation (PBSKP3-124358), NIH Grant R01HD053053 (NICHD & Fogarty International Center), Hawassa University, and Oklahoma State University. We thank Meron Girma and her team at Hawassa University for their technical support.

Table 1: Mean cortisol concentrations and standard deviation are listed for all samples, the interquartile range (25% and 75%) of the samples indicates the distribution, only serum cortisol measurements were skewed, the next row shows the normal ranges for the test kit of R&D and the percentage of samples which were above and below the R&D normal ranges, while all samples were well within the normal ranges in literature (*n* = 39-40).[1]

Cortisol measure-ment	Mean ± SD ng/mL	Interquartile range		R&D test normal range ng/mL	Above R&D %	Below R&D %
Urine	69.0 ± 25.4	48.3	84.9	20.8-153	0	0
Serum	29.1 ± 15.0	18.7	35.5	16.8-75.5	7.5	1.2
Saliva	2.3 ± 1.4	1.2	3.0	0.8-2.9	25	2.5

[1]Mean ± Standard Deviation;

Table 2: Pearson's correlation coefficients between the measurement compartments urine (1), serum (2), and saliva (3) (n = 39-40).[1]

		1	2	3
1	Urine	1.000	-.307	0.359*
2	Serum		1.000	-0.090
3	Saliva			1.000

[1]*<0.05;

Table 3: Pearson's correlation coefficients for cortisol measurements in urine, serum, and saliva with urinary iodine, factors related to inflammation (AGP, CRP, serum albumin), urinary protein, and FRAP (n = 39 - 40).[1]

Cortisol measure-ments	Urinary Iodine	Serum α-1-acid glyco-protein	Serum hs-C-reactive protein	Serum albumin	Urinary total protein	Serum FRAP
Urine	0.504**	-0.062	0.054	0.216	0.414**	0.258
Serum	0.120	0.443**	-0.010	-0.088	-0.018	-0.496**
Saliva	0.057	-0.054	0.304	-0.120	0.093	0.382*

[1]**<0.01; *<0.05.

Table 4: Pearson's correlations coefficients of serum AGP (1), serum CRP (2), serum albumin (3), urinary total protein (4), serum FRAP (5), and urinary iodine (6) (*n* = 39 - 40). [1]

		1	2	3	4	5	6
1	Serum AGP	1.000	0.096	-0.153	-0.010	-0.074	0.048
2	Serum CRP		1.000	-0.430**	-0.122	0.278	-0.010
3	Serum albumin			1.000	0.052	-0.181	0.307
4	Urinary total protein				1.000	0.105	0.298
5	Serum FRAP					1.000	0.055
6	Urinary iodine						1.000

[1]*<0.05; **<0.01.

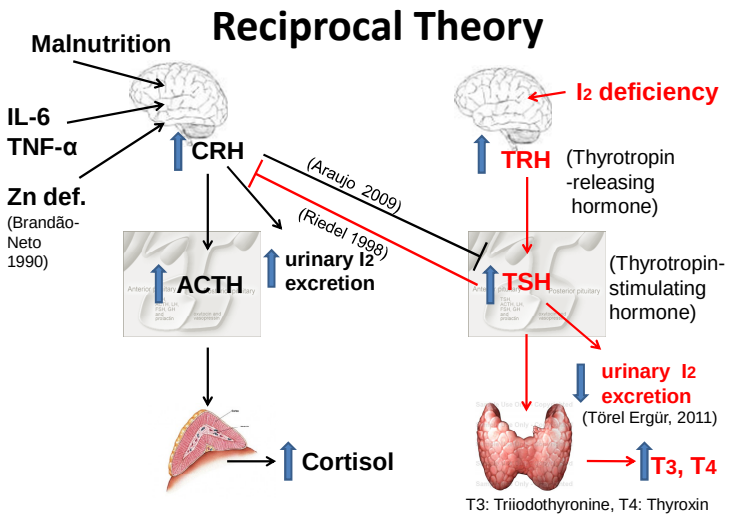

Figure 1: Reciprocal theory based on several studies, indicating the APH axis in black and the HPT axis in red, as well as the interaction between CRH and TSH. Blue arrows up indicate an increase of a substance/concentrations and arrows down indicate a decrease (52, 54-56).

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CHAPTER VI

ZINC SUPPLEMENTATION DID NOT CHANGE BIOCHEMICAL MEASURMENTS IN WOMEN FROM RURAL SIDAMA, SOUTHERN ETHIOPIA

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Abstract

Zinc supplementation trials are seen as an effective technique to assess the extent to which the participants are zinc deficient by determining whether supplements improve health-related biochemical measurements, such as inflammation markers. In this study, a zinc supplementation trial was conducted with volunteers from the Sidama region of Ethiopia. Based on previous dietary and biochemical data from the region, they were assumed to have moderate to severe deficiencies of several nutrients and health indicators.

However, supplementation had no effect on any of the analyzed parameters in blood and urine samples. At baseline, plasma mean (± standard error of the mean) zinc concentration was 704 ± 28 µg/L, near the cutoff of 700 µg/L, while urinary iodine was

far below recommendations of >100 µg/L for nearly all participants, and iron stores were depleted, as indicated by <15 µg/L plasma ferritin in 33.3% of the women. The inflammation markers hsC-reactive protein (21% slightly above normal) and α-1-acid glycoprotein (100% within normal) were in the normal range for most volunteers. Salivary cortisol concentrations were slightly above the ELISA kit reference range in 25% of the volunteers, but all were within the normal range found in literature.

The data were compared with other zinc supplementation trials in order to examine factors contributing to the lack of treatment effect. Studies conducted in vegetarians, vegans and anorexics support the data found in this sample of women who consumed unrefined plant-based diets and very little meat or dairy.

Introduction

Previous research has indicated that the diet of the Sidama region of Ethiopia is composed mainly of unrefined corn bread, fermented enset (*Enset ventricosum*) and kale, with minimal intakes of meat or dairy (1). Dietary zinc and protein intakes would therefore be expected to be very low. In fact, nearly all of the pregnant women in several villages of the Sidama region were found to consume zinc and protein amounts below dietary recommendations. Surprisingly, only about 10% of pregnant women exhibited signs of iron deficiency anemia, although 74% were at risk of low calcium intake based on weighed diet records (2).

Iodized salt is unavailable in the Sidama region (3, 4). In addition, vegetables with goitrogenic effects, such as kale, are consumed on a daily basis; therefore iodine deficiency disorders are prevalent. The goiter rate in women from areas with a similar

altitude and type of diet was found to be near 50%, with mean urinary iodine concentrations of 58.0 µg/L (4).

Fermented foods have a prominent role in the Ethiopian diet, such as in fermented and baked pulp of the enset plant, one of the staples in the Sidama region (5). Fermentation raises B-vitamin content, lowers the pH, and enhances the uptake of zinc, iron, and other dietary minerals depending on the fermentation cultures (6). Despite the positive effect of fermentation on nutrient bioavailability from enset, the daily consumption of high amounts of phytate through unrefined and often unleavened cornbread may decrease zinc uptake (7). Therefore, plasma zinc concentrations would be predicted to be low in the study region. In addition, the dietary absence of meat, a highly bioavailable source of zinc and protein, may contribute further to poor nutrition status (8). Despite the intake of a variety of factors that could impair iron uptake, iron deficiency has not been found to affect a large portion of the study population. This may be because fermentation of enset increased the iron content nearly sixfold, from 1.1 to 6.2 mg/100g, and also increased the calcium content from 52 to 162 mg/100g (1, 9).

For this research project we focused on the incidence of zinc deficiency in Sidaman women. Zinc supplementation studies are increasingly used to assess the prevalence of zinc insufficiency in populations with presumed deficiency (10).The goal of this study was to assess several biomarkers at the baseline and endpoint of a placebo-controlled, double-blind, 17-day zinc supplementation trial. Specifically, we investigated the effect of zinc supplementation on the concentration of zinc, iodine, and ferritin, as well as several stress factors.

Volunteers, materials and methods

Volunteers, study design, supplementation, sample collection

A day before providing their oral consent, volunteers attended an introductory session that explained the goal of the study and what was expected of them as participants. A short screening by a local health worker excluded women with signs of health impairment. Half of the remaining women randomly received a daily supplement of 20 mg zinc, in the form of zinc sulfate (ZinCfant, Nutriset S A S, Malaunay, France), while the other half took a daily dose of placebo (Nutriset S A S, Malaunay, France) for 17 days. The volunteers received the tablets every morning in the health facility of the village. At day one and at day 18 the volunteers provided blood, urine, and saliva samples. For the blood draw, trace mineral free material (Sarsteadt, Newton, NC) was used. All samples were kept on ice in the field and at 4°C or -20°C at Hawassa University in the nearby city of Awassa, where the samples were prepared for transportation to Oklahoma State University either on dry ice or at 4°C.

Zinc in plasma

Blood samples for the assessment of plasma zinc were drawn into lithium-heparin trace mineral free plastic tubes (Sarstedt, Newton, NC). Samples were centrifuged and separated into plasma and packed erythrocytes in the field, and stored at -20°C at Hawassa University. The samples were transported in dry ice to the US laboratory and stored at -80°C thereafter. For analysis of plasma zinc, ICP-MS (SCIEX Elan 9000, Perkin Elmer Life and Analytical Sciences, Norwalk, CT), with gallium as internal standard (Gallium 2%, Perkin Elmer Life and Analytical Sciences, Shelton, CT) was

used. In addition, serum standards (Utak Laboratories Inc., Valencia, CA) were used for quality control. The lower cut off point for adequate plasma zinc in women 20 years and older has been suggested to be 700 µg/L (11).

Iodine in urine

Urinary iodine was measured with the Sandell-Kolthoff reaction method (12) using ammonium persulfate for the digestion (13). Change in color was measured at 405 nm in a spectrophotometer (Beckmann spectrometer DU 800, Fullerton, CA). Standards from 0 to 300 µg/mL were used to interpolate the iodine concentrations of urine samples (GraphPad Software, Inc., La Jolla, CA).

Urinary iodine concentrations are commonly measured in order to assess community levels of deficiency, rather than the iodine status of a single person (14). In this study the iodine status of individual volunteers was categorized according to the four groups defined by the World Health Organization: severe iodine deficiency (median urinary iodine of < 20 µg/L), moderate iodine deficiency (20-49 µg/L), mild iodine deficiency (50-99 µg/L), and adequate iodine nutrition (100-199 µg/L) (14).

Ferritin in serum

For the assessment of serum ferritin of the endpoint samples, an immunoradiometric assay (IRMA F-11, Ramco Laboratories, Inc. Stafford, TX) was used. Tubes with a bead coated with rabbit antihuman spleen ferritin were incubated for 2 hours on a shaker, and then over night at room temperature to allow for full contact with the serum. After rinsing to remove unbound material, the counts of the radiolabeled beads

were quantified in the gamma counter (Cobra II Auto Gamma Counter, Packard, Downers Grove, IL, USA) for 2 minutes each. The calibration curve was calculated using a log transformation for the concentration and a linear scale for the counts (GraphPad Software, Inc., La Jolla, CA). Ferritin concentration in serum and controls was interpolated based on the standard curve. Low iron stores were defined as ferritin concentrations of <15 μg/L (15).

Albumin in serum

Serum albumin was analyzed with an albumin reagent set (BQ 002 CR, BioQuant Diagnostic, San Diego, CA). The color difference is proportional to the albumin concentration and can be measured at 630 nm wavelength in a plate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT). The controls (GEMCAL Reference Serum, Alpha Wassermann Diagnostic Technologies, LLC., West Caldwell, NJ) lay within their reference level. Healthy adults have albumin concentrations between 3.5 and 5.3 g/dL (16).

Cortisol measurement in saliva

The participants were asked to provide a sample of their saliva for the cortisol determination. Each woman chewed on a polyester pad (Salivette, Sarstedt, Newton, NC) that filled with saliva. The sticks were then transferred into their carrier tube and stored on ice. In the laboratory, the test tubes were centrifuged and the saliva transferred to the storage vials. The saliva was frozen and transported on dry ice. Cortisol was measured with a 96-well ELISA plate (Parameter Cortisol KGE008, R&D Systems Minneapolis,

MN). The analysis was performed in a plate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT). Quality controls (Control Set #568 RD6, R&D Systems Minneapolis, MN) were used with low, middle and high cortisol levels. For this study we used the normal reference ranges given by the manufacturer (0.76 to 2.94 ng/mL for salivary cortisol). Concentrations in the literature for healthy adult women, aged 31 to 40 years, range somewhat wider, from 0.94 to 15.14 mg/mL (2.6 to 41.8 nmol/L) (17, 18).

High sensitivity C - reactive protein (CRP) in serum

To examine inflammation in the participants an ELISA test kit (961CRP01H, Helica Biosystems, Inc., Fullerton, CA) was used. The linear standard curve created at 450 nm (Synergy HT, BioTek Instruments, Inc., Winooski, VT) was used to interpolate the sample and control concentrations. The controls (Kentrol Reference serum, Kent Laboratories, Inc. Bellingham, WA) lay within the expected ranges. Mean serum CRP concentrations of 3.8 µg/mL (3.8 mg/L) and below (n=59) were reported by the manufacturer in healthy individuals. For this study, the cutoff for increased CRP was assumed to be ≥3 mg/L (19).

α-1-acid glycoprotein (AGP) in serum

AGP is a hepatic acute phase protein, and concentrations above 1.2 mg/L are suggestive for inflammatory processes. To assess AGP an ELISA quantification kit (40-288-22927F, GenWay Biotech, Inc., San Diego, CA) was utilized. The calibrators, serum (dilution 1:8503) and control (Kentrol Reference serum, Kent Laboratories, Inc. Bellingham, WA) were added to the coated wells. The 96-well plate was then read at 450

nm (Synergy HT, BioTek Instruments, Inc., Winooski, VT). The standard curve was calculated with a four parameter logistic curve fit (GraphPad Software, Inc., La Jolla, CA). The AGP concentrations in serum and controls were interpolated based on the standard curve. The controls lay within the expected range.

Ferric reducing antioxidant power assay (FRAP) in serum

The FRAP assay is based on the method developed by Benzie and Strain in 1996 (2). Briefly, the antioxidant activity in samples is measured by their ability to reduce ferric tripyridyl triazine (Fe III TPTZ) to the ferrous form (Fe II) at a low pH. The ferrous form of iron is blue and can be measured in the spectrophotometer at a wavelength of 593 nm. The method is not specific to any single serum antioxidant (ascorbic acid, uric acid, bilirubin, α-tocopherol, etc.); instead the total antioxidative capacity of the samples is measured. Benzie and Strain reported that healthy adults have FRAP results of 600 to 1600 µmol/L (20).

Statistical analysis

To determine Pearson's correlations coefficients, the statistical software SPSS/PASW 18.0 was used. For statistical assessment of treatment effects, the data were tested for skewedness prior to analysis. Skewed data (cut off more than 3.0 standard deviations away from the mean) were found for urinary iodine and serum CRP, and these variables are expressed as medians. The other data, characteristics of the volunteers and non-skewed biochemical assessments are reported as mean and standard error of the

mean. The effects of the zinc supplementation in both groups (supplemented and placebo) were assessed with a two-factor within-subjects ANOVA (21).

Ethical approval

Approval for this study was given by the ethical committees of Oklahoma State University and Hawassa University. Approval was also granted through two regional government agencies: the South Nations Nationalities and People's State Health Bureau and the Health Office of Awassa. At the national level, approval was granted by the National Health Research Ethics Review Committee of the Ethiopian Science and Technology Commission and the Drug Administration and Control Authority.

Results

Forty women, age 20 to 45 years, of the rural Finichawa area in the Sidama region provided oral consent to participate in a double-blind, placebo-controlled zinc supplementation trial. The mean age of the volunteers was 32.2 ±7.1 years (range 20-45 y). The BMI was 20.5 ± 2.1 (range 15.3-24.2), and five women were underweight (BMIs below <18.5). On average the women had spent 1.4 ± 2.2 years (range 0-10 y) in school, and most were illiterate.

Table 1 indicates the characteristics of the volunteers separated by groups. None of these demographic or anthropometric variables were significantly different at baseline by group. Table 2 indicates the measured biochemical parameters: plasma zinc, urinary iodine, salivary cortisol and serum ferritin, albumin, CRP, AGP, and FRAP. No measurements were significantly different between groups or between time points. The

zinc supplementation had no influence on the plasma zinc concentration of the supplemented group. Group means at all time points were near the 700 µg/L cut off, with a range of 659.0 to 759.2 µg/L (11).

Iodine status was found to be a serious problem in the community. Of the sample group, 42.5% were severely iodine deficient and 32.5% were moderately iodine deficient. Only two of the women (5%) were iodine sufficient. Serum ferritin was assessed only in the endpoint samples, because zinc supplementation with 20 mg zinc for 17 days was not expected to change iron status. In these 40 women, 33.3% were below the cutoff for low iron stores of <15 µg/L (15). Only one woman had serum albumin concentration of 3.3 g/dL, slightly below the range for healthy adults which is 3.5 to 5.3 g/dL (16). Comparing the results for salivary cortisol with the range for healthy adults provided by the ELISA kit (0.76 to 2.94 ng/mL salivary cortisol), around 25% had levels above the given concentrations with a range of 0.04 to 6.60 ng/mL. However, all samples were normal when assuming the reference range found in the literature (18). Based on a cutoff of > 3 mg/L, 18.8% of the volunteers had increased CRP measurements which went up to a maximum of 16.2 mg/L. None of the women had AGP concentrations above the cutoff (>1.2 mg/L), an indicator for increased stress. Approximately half of the volunteers had FRAP concentrations below the normal range of 600 to 1600 µmol/L, with a range of 139-600 µmol/L. The total sample had a range of 139-1077 µmol/L, indicating a below average antioxidant capacity in serum.

Discussion

In this placebo-controlled study of a 17-day supplementation with 20 mg zinc as zinc sulfate, no significant effect on plasma zinc was observed. Due to the fact that the supplements were taken under supervision, the lack of treatment effect could be due to the amount of supplement and/or the timespan of intake. A further reason for the lack of treatment effect could be that to our knowledge most supplementation studies were executed in healthy, well fed study populations. For example a similar study with 25 healthy subjects supplemented with 50 mg zinc as zinc gluconate, well above the upper level of 40 mg zinc per day, reported significantly higher plasma zinc after only 2 days until the end of the supplementation period (18 days) (22). In a second study in which the same supplement was administered to 20 men, plasma zinc was significantly increased after 6 days of supplementation. Their levels decreased slightly at days 10 to 15 of the 15 day supplementation, but stayed significantly above the baseline levels and the levels of the placebo group (23). In an unpublished study by Morejohn and Brown (cited in Hess et al. 2007 (10)), 26 healthy men received a supplement of 15 mg zinc (chemical compound unknown) per day, and within 28 days they showed significant increases in serum zinc concentrations compared to baseline data. A supplementation study with 25 mg zinc per day increased the plasma zinc of healthy adults significantly compared to a control group within 14 days (10, 24).

In addition to these studies showing significant changes in plasma zinc with small supplements of zinc and short time periods, depletion-repletion studies in healthy adults showed a fast reaction of serum or plasma zinc to the dietary intake of zinc (10). However, none of the trials were conducted in previously zinc-restricted populations.

Zinc supplementation studies in pregnant women from developing countries yielded ambiguous results, with some failing to show significant changes despite supplementation over several months (10). In addition, in zinc-deficient populations, comorbidities play an important role. Intervention trials with zinc supplements had no impact on serum zinc concentration when volunteers had multiple deficiencies and when growth was measured for volunteers beyond infancy in Ethiopia (25). Furthermore, there is increasing evidence that serum or plasma zinc concentrations of individuals have limited correlation to zinc intakes and should therefore be analyzed at the population level rather than for the individual (5).

Another fundamental problem associated with zinc deficiency measurement is that there is a certain adaptation to chronic deficient zinc intake which leads to minimal change when measured in serum or plasma (26). In a study with low zinc intake up to 6 months, the adaptation process apparently came into play, which increased the efficiency of zinc uptake from food sources. The zinc homeostasis appeared to be normal when measured in blood (27). Other research using zinc isotopes administered to Chinese women (n = 20) from urban (dietary zinc sufficient, 8.1 mg/d) and rural (marginal dietary zinc deficiency, 5.2 mg/d) areas showed a strong correlation ($r = 0.706$, $p < 0.001$) between total zinc uptake and excretion through the feces. The results from the long-term marginally-deficient women supported the results by Lee et al. (27), who indicated that in deficiency over 6 months, increased uptake was not as important as decreased excretion in maintaining zinc homeostasis (28).

The volunteers in our study had a limited diet based on unrefined plant products with high fiber and phytate content, paired with an extremely low meat intake. Therefore

the data should compare well with research conducted in groups with similar diets. For example, the mean BMI of the Ethiopian volunteers was 20.5 ± 2.1, which compared well with a group of raw food vegetarians (7 women, 11 men, 54.2 ± 11.5 y) eating that specific diet for 1.5 to 10 years (29). The raw food vegetarians had been matched with a similar group eating a typical American diet. The BMI for the raw food vegetarians was significantly lower than for the control, 20.5±2.3 versus 25.4±3.3 (29).

The mean plasma zinc in our group ranged between 659.0 to 759.2 µg/L, while a group of Canadian Seventh Day Adventist women (52.9 ± 15.3 y) had a mean serum concentration of 99 ± 24 µg/dL, despite low zinc, high phytate and fiber intake. In the Canadian study, researchers took precautions not to contaminate the samples by using trace mineral-free supplies (30). The difference in zinc concentration of the samples might be because the amount of minimally processed plant food is probably much higher in the Ethiopian sample, increasing phytate content. Furthermore, dietary diversity in the Ethiopian sample is minimal, further reducing the dietary sources for zinc.

The high dietary intakes of fiber might also explain the low serum iron stores. Approximately 33.3% of our sample had ferritin levels below 15 µg/L; the group mean was 20.2 µg/L. A study in long-term vegans, ovo-lacto vegetarians, and an age-matched control group found ferritin significantly lower in vegans (30.4 ± 4.7 ng/mL) compared to ovo-lacto vegetarians (38.5 ± 3.0 ng/mL), and both groups had serum ferritin significantly below the omnivore controls (55.4 ± 5.3 ng/mL). All means were well above the lower level of 15 mg/L. The study found no correlation between the time span of the diet and iron status (31).

Based on the current literature, no effect on iodine levels due to the zinc supplementation could be expected (32). However, Hess (2010) points out that studies concerned with the relationship between iodine and zinc concentrations have been poorly designed and did not control sufficiently for confounding factors. Thus, the few studies available in hypothyroid subjects had inconclusive results (33).

Most volunteers had albumin concentrations within the normal range of 3.5 -5.3 g/dL, despite a relative low protein intake. A previous study from Ethiopia showed that mean albumin in healthy adults was at 4.2 g/dL, very close to the 4.3 g/dL average in the baseline samples of our study (34). A case report indicated that even in long-term anorexic women, serum albumin stayed normal, except when inflammatory processes occurred (35).

Stress markers like salivary cortisol, serum hsCRP and AGP were within normal ranges for the majority of the volunteers. For example, only 25% our sample of Ethiopian women had salivary cortisol concentrations slightly above the manufacturer's normal range of 0.76 to 2.94 ng/mL. However, all were within the normal range found in the literature 0.94 to 15.14 mg/mL (2.6 to 41.8 nmol/L) (18). Mostly normal salivary cortisol concentrations were also found in 61 hospitalized anorexic girls and boys (mean age 14.9 y, BMI at admission 15.3) with 98% in the normal range (36).

In vegetarians consuming no fish or meat and with very limited milk and dairy intake, hs CRP was significantly lower (0.77 ± 1.29 mg/L) than the non-vegetarian control group (1.30 ± 1.38 mg/L) (37). These results are comparable to those from adults eating a raw food diet, where CRP reached 0.6 ± 0.8 mg/L, while the control had 1.8 ± 2.4 mg/L (29).

The one factor which was strikingly different between the Ethiopian volunteers and the vegetarians found in the literature was the persistently low FRAP level of our sample. Long-term vegetarians (27 women, 3 men, 44.2±9.0 y) did not have diminished FRAP levels compared to an age-matched non-vegetarian group (37). Our sample had only half as much FRAP activity compared to an ovo-lacto vegetarian group from Hong Kong, indicating diminished antioxidant activity. However, our sample was consistent with a sample of 24 female and male vegetarian Slovaks (half of which were ovo-lacto vegetarians, half lacto-vegetarians) who had FRAP levels of 766.5 ± 39.0 µmol/L (38).

In conclusion, no treatment effect could be found in blood, saliva and urine parameters of Ethiopian women supplemented with 20 mg zinc per day for 17 days. One possible explanation is that the amount of zinc or the time span of the supplementation was limiting. Another is that plasma zinc and other measured parameters may not be sensitive measures of zinc status in these women. Remarkably, the iodine levels in this group of women were very low. Comparing the biochemical parameters of our sample with the literature on vegetarians, the inflammation markers were within usual levels. The antioxidant capacity of the Ethiopian sample was somewhat lower than expected, based on the literature.

One difficulty in interpreting our data is the lack of studies in non-pregnant women with the same type of diet as our study group. Pregnant women from the study region, women with restricted western diets (such as vegans and raw food vegetarians), and anorexic westerners may not serve well as comparisons for our sample. Pregnant women have higher intake needs and lower plasma concentrations of some indicators due to hemodilution. In addition, even restricted western diets are most likely still more

diverse in their food choices than the diets consumed by our volunteers. Furthermore, in developed countries the intake of dietary supplements is common.

This research was supported by the Swiss National Science Foundation (PBSKP3-124358), NIH Grant R01HD053053 (NICHD & Fogarty International Center), Hawassa University, and Oklahoma State University. We thank Meron Girma and her team at Hawassa University for their technical support.

Table 1: Mean and standard deviation of the mean (Mean ± SEM) of baseline characteristics of women: age (y), height (m), weight (kg), BMI (kg/m^2), education (y in school) by group, n = 20.

	Placebo	Supplement
Age	33.8 ± 1.6	30.6 ± 1.5
Height (m)	1.56 ± 0.2	1.58 ± 0.2
Weight (kg)	50.4 ± 1.7	51.5 ± 1.6
BMI (kg/ m^2)	20.2 ± 0.4	20.8 ± 0.5
Education (y in School)	1.5 ± 0.6	1.2 ± 0.5

Table 2: Biochemical measurements of the placebo and zinc supplemented groups (means ± standard deviation of the mean or median ± standard deviation) (*n* = 16-20).[1,2,3]

		Placebo		Zinc supplement	
		Baseline	Endpoint	Baseline	Endpoint
Plasma zinc	µg/L	703.7 ± 28.1	659.0 ± 28.7	759.2 ± 24.8	718.9 ± 23.7
Urinary iodine†	µg/L	29.5 ± 21.4	17.1 ± 31.3	16.7 ± 35.0	22.78 ± 45.9
Serum ferritin	µg/L	‡	17.47 ± 1.35	‡	22.8 ± 3.0
Serum albumin	g/dL	4.34 ± 0.06	4.13 ± 0.11	4.3 ± 0.11	4.2 ± 0.09
Salivary cortisol	ng/mL	2.42 ± 0.29	1.88 ± 0.23	2.66 ± 0.45	2.36 ± 0.41
Serum hsCRP†	mg/L	1.04 ± 3.68	0.63 ± 0.75	1.27 ± 4.66	0.81 ± 2.79
Serum AGP	mg/mL	0.37 ± 0.04	0.17 ± 0.03	0.34 ± 0.05	0.19 ± 0.03
Serum FRAP	µmol/L	560.2 ± 64.3	470.0 ± 55.7	646.0 ± 32.7	541.7 ± 55.7

[1]* $p<0.05$;
[2]† median ± standard deviation;
[3]‡ only endpoint samples for ferritin were analyzed.

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CHAPTER VII.

ZINC SUPPLEMENTATION REDUCED DNA BREAKS MEASURED BY COMET ASSAY IN ETHIOPIAN WOMEN FROM SIDAMA

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Abstract

Assessment of zinc status remains a challenge, despite the use of sensitive techniques for measuring plasma zinc concentrations. The comet assay has been demonstrated to successfully assess the effect of changes in zinc intake due to supplementation on a sub cellular level in humans (1). However, this method had not yet been validated with samples obtained in the field environment of a developing country.

For this study, 40 Ethiopian women received 20 mg zinc as zinc sulfate or a placebo daily for 17 days. Baseline and endpoint whole blood samples were analyzed by comet assay, and plasma zinc was analyzed by inductively coupled plasma mass

spectrometry (ICP-MS). Comet assay slides were scored by investigators blinded to the treatments.

Plasma zinc concentrations did not change significantly between baseline and endpoint in either group ($p > 0.05$). Although zinc supplementation did not alter plasma zinc concentrations, the additional zinc did statistically decrease comet measurements at endpoint. Tail length ($p = 0.004$), tail moment ($p = 0.008$), total intensity ($p = 0.007$), total area ($p = 0.001$) and width ($p = 0.019$) were significantly decreased in endpoint samples, indicating fewer DNA breaks within the supplemented individuals. Three measurements showed significant differences between the groups: tail moment ($p = 0.038$), total area ($p = 0.007$) and total intensity ($p = 0.043$). These results are in agreement with other studies (1, 2), suggesting that the comet assay is a valid method to assess the effects of dietary zinc intake on DNA integrity.

Introduction

Zinc deficiency is a worldwide health problem that especially affects populations consuming plant-based diets in developing countries. Due to the numerous dietary and non-dietary factors affecting zinc bioavailability, the assessment of human zinc status is important. However, no simple biochemical analysis is available that represents systemic zinc status. Zinc status is often assessed by measuring the amount of zinc in red blood cells, leukocytes, neutrophils, or plasma, although each of these compartments is subject to regulation independent of zinc status and may not provide accurate results (3). As a result of the challenges associated with determining an individual's zinc status, global prevalence of zinc deficiency is assessed with plasma zinc (3, 4).

The comet assay is an indirect measurement reflecting changes in zinc intake on a subcellular level. The assay showed promising results in a zinc restriction and repletion study in men, reflecting the effect of zinc on DNA integrity (1). This micro-electrophoretic technique was first introduced in 1984 (5) and later improved by Singh (6), and has been used in cancer research as well as in the field of genetic toxicology. Essentially, the technique allows investigators to visualize and quantify the extent of DNA damage within single, agar embedded cells. In this electrophoresis assay, intact DNA is retained in the "head", while fragmented DNA forms the tail, resulting in the formation of so-called "comets" (7). The comets are visualized and can be measured using fluorescence microscopy (8). Due to the essential role of zinc in DNA replication and repair mechanisms, changes in zinc availability may contribute to changes in the comet size (3).

The goal of this study was to assess the effects of a double-blind, placebo-controlled supplementation trial with 20 mg zinc as zinc sulfate. The 40 women from Ethiopia received a zinc supplement or placebo for 17 days. At baseline and endpoint, plasma zinc was measured and DNA integrity was assessed through comet assay.

Volunteers, materials and methods

Volunteers, study design, supplementation, sample collection

The day before giving oral consent the volunteers attended an orientation in which local research staff from Hawassa University explained the goals of the study and what was involved with participation. Forty non-pregnant women, age 20 to 45 years, from the Finichawa area in the Sidama region of Ethiopia, were randomly assigned into two

groups. A healthcare assistant excluded volunteers with illness, inflammation, or other obvious signs of impaired health.

The volunteers received a total of 20 mg of zinc as zinc sulfate (ZinCfant, Nutriset S A S, Malaunay, France), or a placebo tablet (Nutriset S A S, Malaunay, France) for 17 days. Blood samples were drawn in lithium-heparin plastic tubes designed for trace-element research (Sarstedt, Newton, NC). For the comet assay the whole blood was stored on ice in the field and during transportation to the USA. For the plasma samples, an aliquot of the blood was centrifuged, and plasma and packed erythrocytes were stored on ice in the field and frozen at -20°C in the laboratory. The plasma and erythrocytes were transported on dry ice to the USA, where they were stored at -80°C.

Zinc in plasma

Plasma was analyzed by inductively coupled plasma mass spectrometry (ICP-MS) (SCIEX Elan 9000, Perkin Elmer Life and Analytical Sciences, Norwalk, CT) with gallium as internal standard (Gallium 2%, Perkin Elmer Life and Analytical Sciences, Shelton, CT). Serum standards (Utak Laboratories Inc., Valencia, CA) were used for quality control. The lower cut off point for plasma zinc in adult women has been suggested as 700 µg/L or 10.65 µmol/L (2).

Comet assay

Alkaline single cell gel-electrophoresis was used to assess single-strand breaks (6). The method employed for sample preparation is described by Song et al. (1). Due to minor hemolysis the method was slightly modified. The wash step was omitted, and

instead the blood samples were centrifuged and the cell sediment was used for the comet assay. Furthermore, the cells were stored in lysis buffer for one hour only. The alkalized cells underwent electrophoresis using exactly the same conditions for each batch. The analysis of the electrophoresis slides was performed at Oregon State University under the supervision of Dr. Emily Ho as described by Song et al. (1). The analysts were blinded to the treatments.

Statistical analysis

Treatment effects were analyzed using analysis of variance with repeated measures (PROC MIXED) with an autoregressive period 1 error structure. The simple effect of treatment given time (SLICE option in an LSMEANS statement) was used to identify significant changes from baseline with supplementation using SAS (SAS Institute, Inc., Cary, NC, USA). Results were expressed as means ± standard error of the mean.

Ethical approval

Approval for this study was given by the ethical committees of Oklahoma State University and Hawassa University. Approval was also granted through two regional government agencies: the South Nations Nationalities and People's State Health Bureau, and the Health Office of Awassa. At the national level, approval was granted by the National Health Research Ethics Review Committee of the Ethiopian Science and Technology Commission, and the Drug Administration and Control Authority.

Results

There were no significant differences in plasma zinc concentrations within control or supplemented groups between baseline and endpoint after 17 days of supplementation ($p > 0.05$) (Table 1).

Significant differences between baseline and endpoint comet measurements were found for most parameters in the supplemented group (Table 2). In the placebo group only the tail length decreased significantly between baseline and endpoint.

Discussion

The plasma zinc concentrations of the Ethiopian women were higher than expected for this region of Ethiopia. Studies from the Sidama region reported mean plasma zinc (± standard deviation of the mean) in pregnant women of 450.5 ± 80.1 µg/L (6.86 ± 1.22 µmol/L) (10). However, another study from the Gondar region, Ethiopia, found a mean plasma zinc concentration (± standard deviation) of 882 ± 342 µg/L in healthy subjects (31.0 ± 6.5 years of age), indicating large differences within the country (11). The plasma zinc in our study was near the lower cut-off level for deficiency of 700 µg/L (10.65 µmol/L) for non-pregnant adults (12).

Assessment of zinc is difficult and current methods are not always able to reflect true status on an individual level (13). However, zinc supplementation trials are considered one of the best methods to identify groups at risk for zinc deficiency (14). The assessment of plasma zinc in the study sample provided an example of the problem with using plasma zinc as a zinc status indicator. The mean zinc concentration of the

supplemented group did not change significantly despite supervised intake of the zinc supplement.

Zinc deficiency has a negative impact on cell integrity, which was demonstrated in numerous studies. Deficiency and repletion of zinc for example, influenced the DNA integrity of blood cells in a rat model (15). Research in lung fibroblasts clearly demonstrated that the deficiency in zinc led to higher levels of oxidation in the cells and consequently to increased DNA damage, the latter assessed by comet assay (16). Zinc however, is also part of the repair mechanisms. First, zinc as part of copper-zinc superoxide dismutase (CuZn SOD) decreases the effect of oxidative stress. Second, zinc improves and increases repair mechanisms as part of p53 and apurinic endonuclease (APE), two proteins that are involved in cell signaling. Thirdly, APE has a double function of being a repair protein and controlling DNA-binding of several transcription factors (17). Due to zinc's importance for DNA integrity, the comet assay can be a valid tool to assess changes of zinc intake (18).

Research demonstrated that changes in the comet measurement called tail moment are highly significant, based on changes in zinc intake in humans (1) and in animal models (15, 19). In our sample the tail moment of the supplemented group decreased 1.2 fold during the17-day supplementation period. This is similar to the 1.3 fold increase described by Song et al. in a rat study, where rats experienced marginal zinc deficiency (15), while the tail moment measurements of the placebo group changed only marginally, with a non-significant 1.05 fold decrease compared to baseline values. The tail moment is a calculated measurement expressing the percentage of migrated DNA in the tail multiplied by the length between the midpoint of the head and the tail (20). In addition to

the tail moment measurement, in our study several other comet assay measurements showed significant differences between the baseline and endpoint samples in the supplemented group, supporting the tail moment results (Table 2).

The comet assay method specified stable conditions during sampling and rapid preparation of slides in order to prevent artificial DNA breaks (21). Despite that recommendation, with this study we showed that it is possible to have significant results from samples gained in a field study abroad and transported to the laboratory in the USA.

In conclusion, this study demonstrated that the comet assay is a useful tool to identify DNA breaks due to changes in dietary zinc intake. The zinc supplementation trial also revealed that plasma zinc concentrations did not change significantly after a 17-day supplementation with 20 mg of zinc.

A limitation of this study is that due to storage and transportation over several days, the samples were somewhat damaged, which increased the DNA break measurements in all samples compared to freshly prepared comet slides. However, the highly significant decrease of DNA breaks in the zinc supplemented group, without change in the placebo group, demonstrated the treatment effect.

This research was supported by the Swiss National Science Foundation (PBSKP3-124358), NIH Grant R01HD053053 (NICHD & Fogarty International Center), Hawassa University, Oregon State University and Oklahoma State University. We thank Meron Girma and her team at Hawassa University for their technical support.

Table 1: Effect of zinc supplementation on plasma zinc concentrations (µg/L) in Ethiopian women, receiving 20 mg zinc as zinc sulfate or placebo daily for 17 days.[1]

		Plasma zinc (µg/L)		
Zinc status	*n*	Baseline	*n*	Endpoint
Supplemented	17	759.2 ± 24.9	20	718.2 ± 23.7
Placebo	20	703.7 ± 28.1	20	659.0 ± 28.7

[1] Values are means ± standard error of the mean.

Table 2: Effect of zinc supplementation on different comet measurements (tail length, tail moment, tail intensity, total area, total intensity, width) from blood cells from a sample of Ethiopian women.[1,2,3]

	Supplemented, *n* = 20		Placebo, *n* = 18	
	Baseline	Endpoint	Baseline	Endpoint
Tail length	151.7 ± 1.7**	143.7 ± 1.5**	154.0 ± 2.5*	147.4 ± 1.7*
Tail moment	38.6 ± 2.3**	30.7 ± 1.6**†	39.3 ± 2.4	37.3 ± 2.4†
Tail intensity	62.7 ± 2.7	57.0 ± 2.1	62.7 ± 3.0	62.8 ± 3.4
Total area	5657.9 ± 227.8**	4910.4 ± 127.2**††	5861.9 ± 194.1	5626.3 ± 157.1††
Total intensity	239,368 ± 8387**	214,029 ± 7188**†	240,746 ± 7329	236.794 ± 8083†
Width	64.3 ± 1.6*	60.48 ± 0.8*	66.3 ± 1.6	63.4 ± 1.0

[1] Values are means ±standard error of the mean.
[2] Significant differences from baseline within treatment, illustrated by **<0.01; *<0.05.
[3] Significant differences between supplemented and placebo group at the same time point, illustrated by ††<0.01; †<0.05.

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CHAPTER VIII

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Summary of the findings

The purpose of this study was to assess plasma zinc and other health indicators in serum and urine, as well as acute phase proteins before and after a double-blind placebo-controlled zinc supplementation trial in Southern Ethiopia. A further goal was to assess changes in the mRNA expression of the Zip 4 (SLC39A4) zinc transporter in buccal cells due to the zinc supplementation. In addition, cortisol levels measured in saliva, urine and serum were compared. The final goal was to study the change in antioxidant capacity with the ferric reducing antioxidant power (FRAP) method and oxidative stress with the comet assay.

Null hypotheses

$H0_1$: There will be no significant difference between plasma zinc of the supplemented and placebo groups at baseline and after 17 days of treatment with 20 mg zinc taken as daily tablets.

There was no significant difference in the plasma zinc concentration before and after the zinc supplementation so the null hypothesis was accepted.

$H0_2$: There will be no significant difference in the health indicators (serum ferritin, and albumin, urinary iodine, and total protein) or acute phase proteins (hsC-reactive protein, α-1-acid glycoprotein) in either group at baseline and endpoint of the trial.

There was no significant difference in any of the measured biochemical parameters at baseline and endpoint for either group; therefore the null hypothesis was accepted. No treatment effects were found after the supplementation for 17 days with 20 mg zinc daily.

$H0_3$: There will be no significant change in the expression for Zip 4 transporter (SLC39A4) mRNA and metallothionein mRNA due to zinc supplementation in Ethiopian women.

Despite positive test results of the method, the measurement could not be successfully performed in samples from Ethiopia, due to the fact that the sample RNA could not be extracted. Therefore, the hypothesis could not be tested.

$H0_4$: There will be no correlation between cortisol measured in saliva, serum, and urine samples. In addition, there will be no significant change in cortisol comparing baseline to endpoint in either group.

The salivary and urinary cortisol values were significantly correlated to each other, so the null hypothesis was rejected. However serum cortisol measurements were not correlated with either salivary or urine measurements and zinc supplementation had no effect on cortisol concentrations in any compartment.

$H0_5$: There will be no significant antioxidative effect due to the zinc supplement, measured with FRAP or with the oxidative stress in comet assay.

Zinc supplementation significantly decreased DNA breaks measured by comet assay so the null hypothesis was rejected. However, no changes in serum antioxidative capacity were detected in either group.

Conclusions

The placebo- controlled supplementation trial with 20 mg of zinc as zinc sulfate daily had no significant effect on plasma zinc concentrations in this 17 day trial. Furthermore, none of the additional health parameters (urinary iodine, serum ferritin, serum and urine total protein, serum albumin) and neither of the chosen acute phase proteins (serum C-reactive protein, serum α-1-Acid Glycoprotein) changed significantly due to the supplementation.

This study identified Zip4 and MT mRNA in buccal cells, and showed that there was a negative relationship between dietary zinc intake, and Zip4 RNA expression in buccal cells in small laboratory tests. However, with the established method, mRNA could not be extracted for the quantitative PCR method.

The researcher found no correlation between serum and saliva cortisol; however, a weak correlation between urinary and saliva measurements was established. Based on this study, cortisol did not correlate to other stress measurements.

The study showed that zinc supplementation had a highly significant impact on the integrity of the cell DNA obtained in whole blood; however no difference in total antioxidant concentrations could be detected based on the FRAP method.

Overall, the research showed that the women who volunteered for this study were not as zinc deficient as was assumed, based on data from the region. However the mean plasma zinc concentration was just marginally above the lower cutoff for zinc adequacy. Acute phase proteins and stress parameters were not or only slightly above normal ranges. However, the overall iodine level showed moderate iodine deficiency for this group.

Recommendations for further research

With the exception of their low urinary iodine, the general health of the Sidaman volunteers was not as bad as expected based on the literature. However, interventions are necessary in order to establish more dietary diversity in this village. The women need to be educated about their general health, and to have access to a source of iodine to prevent health impairment for themselves, their families, and their offspring.

Based on the literature and these research findings, a quantitative assessment of the zinc transporter Zip4 and possibly MT mRNA in buccal cells with the suggested method is likely. Due to the fact that an increase in dietary zinc is not necessarily reflected in plasma zinc levels in individuals, plasma zinc might not be the measurement to which the findings of a quantitative PCR assessment should be compared.

For future zinc supplementation trials, a different regime needs to be employed. Possible changes would be an increase in the zinc concentration, an expanded timeframe, intermittent blood draws, and a larger group of volunteers.

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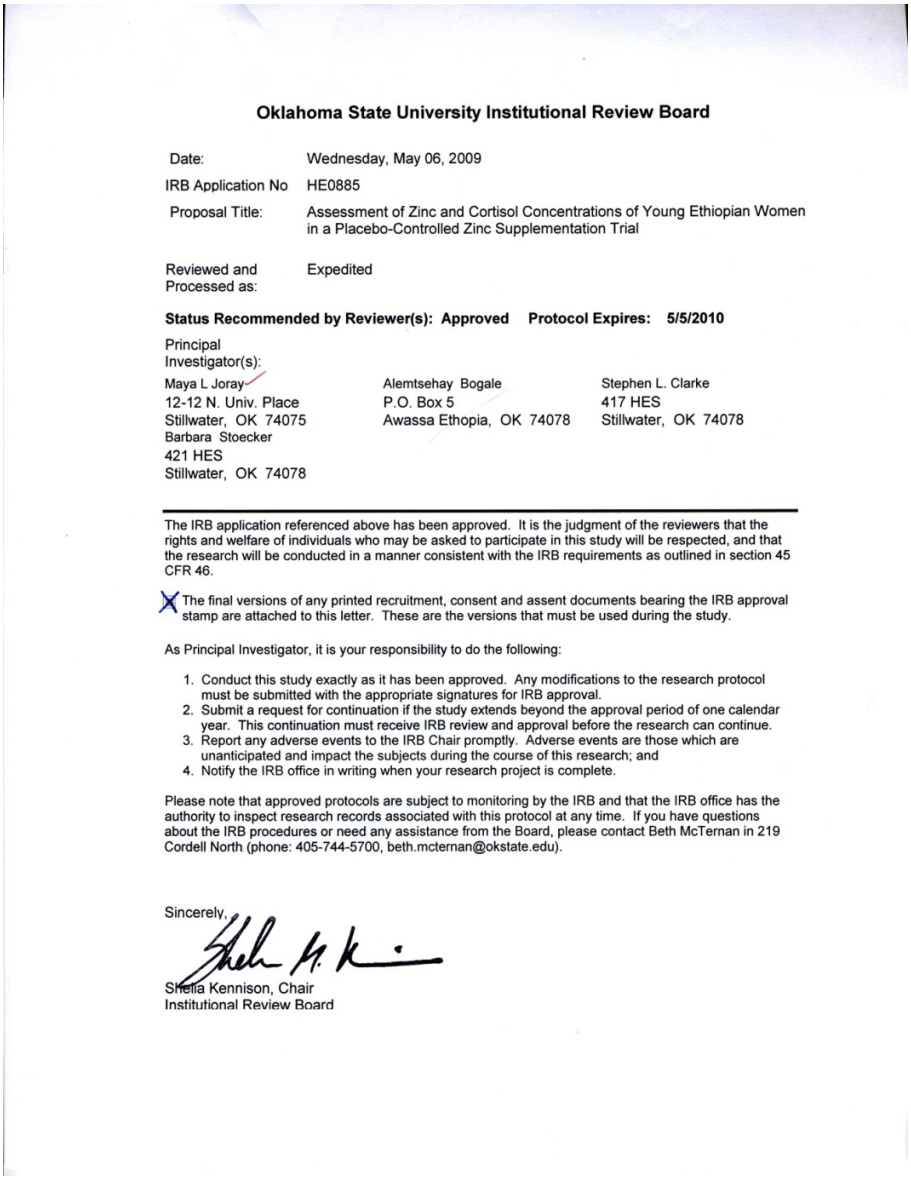
X. APPENDICES

Appendix A

Approval from Oklahoma State University Institutional Review Board, Oklahoma, USA



Oklahoma State University Institutional Review Board

Date:	Wednesday, May 06, 2009
IRB Application No	HE0885
Proposal Title:	Assessment of Zinc and Cortisol Concentrations of Young Ethiopian Women in a Placebo-Controlled Zinc Supplementation Trial
Reviewed and Processed as:	Expedited

Status Recommended by Reviewer(s): Approved Protocol Expires: 5/5/2010

Principal Investigator(s):

Maya L Joray
12-12 N. Univ. Place
Stillwater, OK 74075

Alemtsehay Bogale
P.O. Box 5
Awassa Ethopia, OK 74078

Stephen L. Clarke
417 HES
Stillwater, OK 74078

Barbara Stoecker
421 HES
Stillwater, OK 74078

The IRB application referenced above has been approved. It is the judgment of the reviewers that the rights and welfare of individuals who may be asked to participate in this study will be respected, and that the research will be conducted in a manner consistent with the IRB requirements as outlined in section 45 CFR 46.

X The final versions of any printed recruitment, consent and assent documents bearing the IRB approval stamp are attached to this letter. These are the versions that must be used during the study.

As Principal Investigator, it is your responsibility to do the following:

1. Conduct this study exactly as it has been approved. Any modifications to the research protocol must be submitted with the appropriate signatures for IRB approval.
2. Submit a request for continuation if the study extends beyond the approval period of one calendar year. This continuation must receive IRB review and approval before the research can continue.
3. Report any adverse events to the IRB Chair promptly. Adverse events are those which are unanticipated and impact the subjects during the course of this research; and
4. Notify the IRB office in writing when your research project is complete.

Please note that approved protocols are subject to monitoring by the IRB and that the IRB office has the authority to inspect research records associated with this protocol at any time. If you have questions about the IRB procedures or need any assistance from the Board, please contact Beth McTernan in 219 Cordell North (phone: 405-744-5700, beth.mcternan@okstate.edu).

Sincerely,

Sheila Kennison, Chair
Institutional Review Board

Appendix B

Approval from Oklahoma State University Institutional Review Board: Extension, Stillwater, Oklahoma, USA

Oklahoma State University Institutional Review Board

Date	Friday, October 30, 2009	Protocol Expires:	5/5/2010
IRB Application	HE0885		
Proposal Title:	Evaluation of new Techniques for Zinc and Cortisol Assessment with a Placebo-Controlled Zinc Supplementation Trial in a Subsample of Young Ethiopian Women		
Reviewed and Processed as:	Expedited **Modification**		

Status Recommended by Reviewer(s) **Approved**

Principal Investigator(s) :

Maya L Joray	Stephen L. Clarke	Meron Girma
12-12 N. Univ. Place	417 HES	Hawassa University
Stillwater, OK 74075	Stillwater, OK 74078	Hawassa, Ethiopia, OK

The requested modification to this IRB protocol has been approved. Please note that the original expiration date of the protocol has not changed. The IRB office MUST be notified in writing when a project is complete. All approved projects are subject to monitoring by the IRB

X The final versions of any printed recruitment, consent and assent documents bearing the IRB approval stamp are attached to this letter. These are the versions that must be used during the study.

Signature :

Shelia Kennison, Chair, OSU Institutional Review Board

Friday, October 30, 2009
Date

Appendix C

Approval from Hawassa University, College of Health Sciences, Awassa, Ethiopia

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Hawassa University
College of Health Science

Ref No HSC/2510/01

Date 28 - 08 - 01

To : AVP-REP Office

From : College of Health Sciences

Subject: **Ethical Clearance**

The Institutional Research Review Committee of College of Health Sciences, Hawassa University, on its meeting of 28/04/09 has reviewed the following research proposal entitled,

" Zinc, Iron, Iodine status and Cognitive Function in Women from Sidama Zone, SNNPR, with Evaluation of New Technique of Zinc Assessment" by Mr. Tafere G/Egziabher (staff of Hawassa University who is on a study leave for masters program).

The proposal was reviewed as per the National Research Review Guidelines and approved for ethical clearance. However, the final ethical approval is required from the NERC. Hence, the purpose of this letter is to request your office facilitate the process of ethical clearance at the national level.

Kind Regards!

DR. Yifru Berhan Mitke
Head, College of Health Science

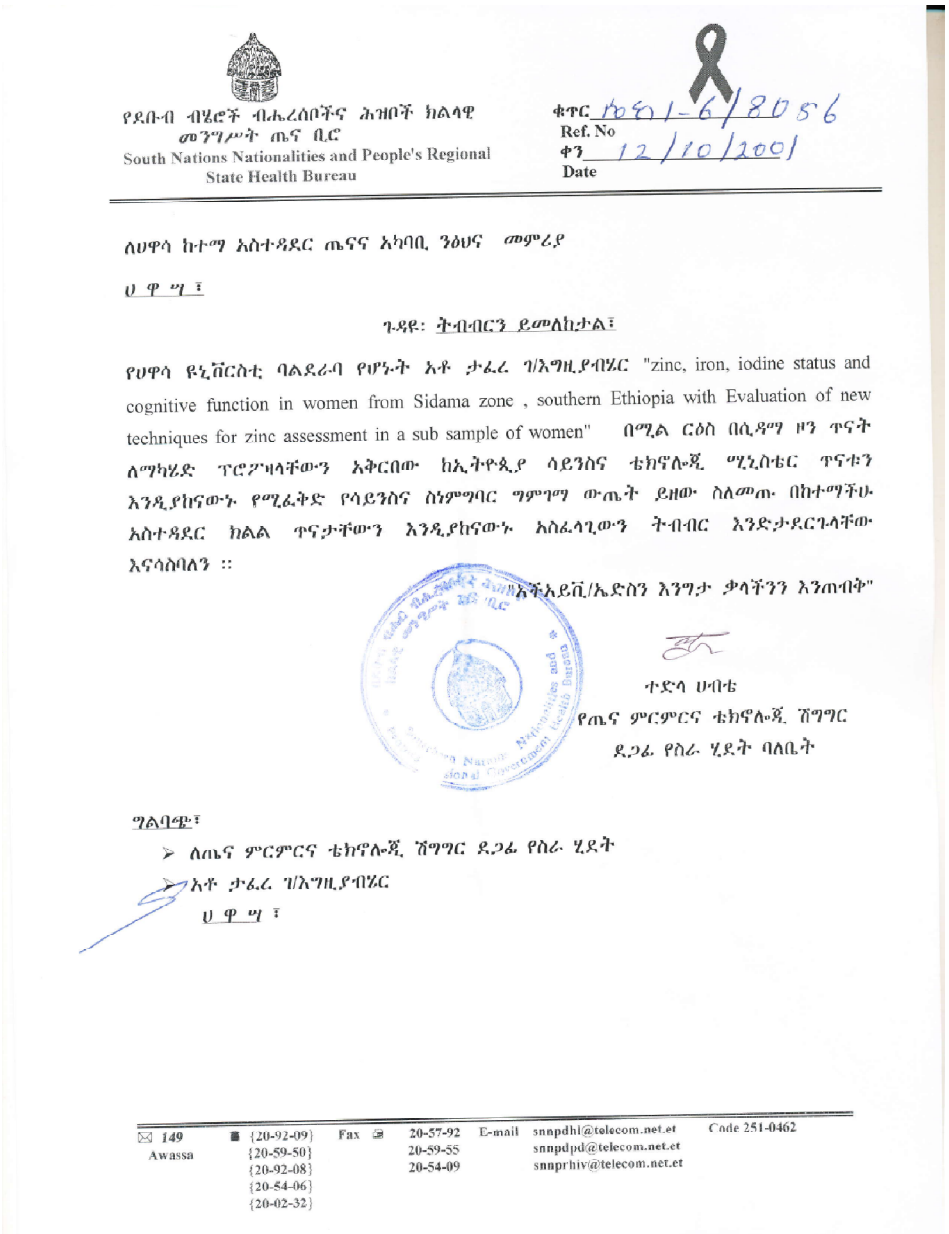
Cc
Mr. Tafere G/Egziabher
AQA

Research Extension Office
CHS

☎ + 046-220-87-55 Fax + 046 20-87-55 e-mail aca @ telecom.net.et
✉ 1560 Awassa, Ethiopia

Appendix D

Approval from the South Nations Nationalities and People's State Health Bureau, Awassa, Ethiopia



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South Nations Nationalities and People's Regional State Health Bureau

ቁጥር ጤቢ1-6/8056
Ref. No
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ጉዳዩ፡ ትብብርን ይመለከታል፣

የሀዋሳ ዩኒቨርስቲ ባልደራባ የሆኑት አቶ ታፈረ ገ/እግዚያብሄር "zinc, iron, iodine status and cognitive function in women from Sidama zone , southern Ethiopia with Evaluation of new techniques for zinc assessment in a sub sample of women" በሚል ርዕስ በሲዳማ ዞን ጥናት ለማካሄድ ጥሮያዣላቸውን አቅርበው ከኢትዮጲያ ሳይንስና ቴክኖሎጂ ሚኒስቴር ጥናቱን እንዲያከናውኑ የሚፈቅድ የሳይንስና ስነምግባር ግምገማ ውጤት ይዘው ስለመጡ በከተማችሁ አስተዳደር ክልል ጥናታቸውን እንዲያከናውኑ አስፈላጊውን ትብብር እንድታደርጉላቸው እናሳስባለን ::

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Fax 20-57-92 20-59-55 20-54-09
E-mail snnpdhl@telecom.net.et snnpdpd@telecom.net.et snnprhiv@telecom.net.et
Code 251-0462

Appendix E

Approval from the Awassa Health Office, Awassa, Ethiopia

Awassa T.C.A.H.T.C Health Office

ቁጥር 1125/HCC/2001

ቀን 13-10-2001

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ጉዳዩ፡- ትብብር ስለመጠየቅ

የሀዋሳ ዩኒቨርስቲ ባልደረባ የሆኑት አቶ ታፈረ ገ/እግዚአብሔር "Zinc iron Iodine Status and Cognitive function in women from sidama zone" በሚል ርዕስ ላይ ጥናት ለማካሄድ የእናንተን ቀበሌ የመረጡ ስለሆነ በሚያስፈልጋቸው ሁሉ ቀና ትብብር ታደርጉላቸው ዘንድ እናሳስባለን

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Kebede Keshela Kia

H/T/S/C Health office Head

ግልባጭ

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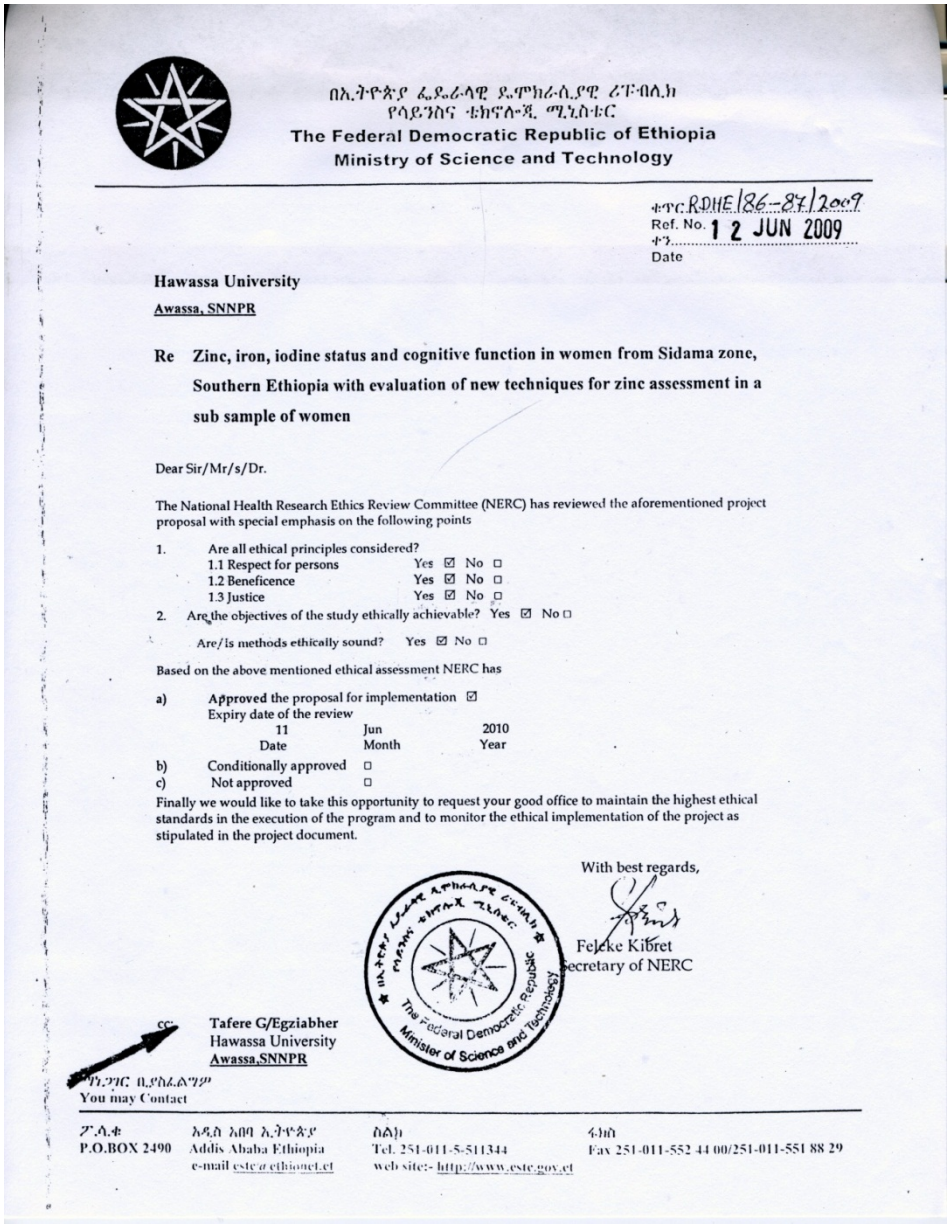
Appendix F

Approval from the Federal Democratic Republic of Ethiopia, Ministry of Science and Technology, Addis Ababa, Ethiopia



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The Federal Democratic Republic of Ethiopia
Ministry of Science and Technology

ቁጥር Ref. No. RDHE/86-87/2009
ቀን Date 12 JUN 2009

Hawassa University
Awassa, SNNPR

Re Zinc, iron, iodine status and cognitive function in women from Sidama zone, Southern Ethiopia with evaluation of new techniques for zinc assessment in a sub sample of women

Dear Sir/Mr/s/Dr.

The National Health Research Ethics Review Committee (NERC) has reviewed the aforementioned project proposal with special emphasis on the following points

1. Are all ethical principles considered?
   - 1.1 Respect for persons — Yes ☑ No ☐
   - 1.2 Beneficence — Yes ☑ No ☐
   - 1.3 Justice — Yes ☑ No ☐
2. Are the objectives of the study ethically achievable? Yes ☑ No ☐

   Are/is methods ethically sound? Yes ☑ No ☐

Based on the above mentioned ethical assessment NERC has

a) **Approved** the proposal for implementation ☑
   Expiry date of the review
   11 (Date) Jun (Month) 2010 (Year)
b) Conditionally approved ☐
c) Not approved ☐

Finally we would like to take this opportunity to request your good office to maintain the highest ethical standards in the execution of the program and to monitor the ethical implementation of the project as stipulated in the project document.

With best regards,

Feleke Kibret
Secretary of NERC

The Federal Democratic Republic Minister of Science and Technology

cc: Tafere G/Egziabher
Hawassa University
Awassa,SNNPR

ሊያገኙኝ ቢያስፈልግዎ
You may Contact

ፖ.ሳ.ቁ P.O.BOX 2490 — አዲስ አበባ ኢትዮጵያ Addis Ababa Ethiopia — e-mail estc@ethionet.et
ስልክ Tel. 251-011-5-511344 — web site:- http://www.estc.gov.et
ፋክስ Fax 251-011-552 44 00/251-011-551 88 29

Appendix G

Approval from the Federal Democratic Republic of Ethiopia, Drug Administration and Control Authority, Addis Ababa, Ethiopia

በኢትዮጵያ ፌዴራላዊ ዴሞክራሲያዊ ሪፐብሊክ
THE FEDERAL DEMOCRATIC REPUBLIC OF ETHIOPIA
የኢትዮጵያ የመድኃኒት አስተዳደርና ቁጥጥር ባለሥልጣን
DRUG ADMINISTRATION AND CONTROL AUTHORITY OF ETHIOPIA

ቁጥር Ref. No. 07/6/27/46

ቀን Date 29 JUN 2009

To Tefere G/Egziabher
Hawassa University
Hawassa

Dear Sirs,

We refer your application dated June 22, 2009 for authorization of clinical study (research) entitled "Zinc, iron, iodine status and cognitive function in women aged 18 years and above in Sidama zone, Southern Ethiopia with evaluation of new techniques for zinc assessment in a sample of women."

Please find four pages of our assessment report. We reconsider your application after you submit your documentation according our assessment report.

Best Regards,

ዳይሬክተር
NIT MENGISTU
Director, Product Registration &

Cc: Product Registration and Licensing Directorate
DACA

VITA

Maya Lucia Joray

Candidate for the Degree of

Doctor of Philosophy or Other

Thesis: EVALUATION OF NEW TECHNIQUES FOR ZINC AND CORTISOL ASSESSMENT WITH A PLACEBO-CONTROLLED ZINC SUPPLEMENTATION TRIAL IN A SUBSAMPLE OF ETHIOPIAN WOMEN

Major Field: Human Environmental Sciences/Nutrition

Biographical:

Personal Data:
Second prize poster winner in OSU Research Symposium in Biological and Biomedical Science
Winterfeldt Graduate Scholarship recipient

Education:
Completed the requirements for the Doctor of Philosophy in Nutritional Sciences at Oklahoma State University, Stillwater, Oklahoma in July, 2011.
Completed the requirements for the Master of Science Degree in International Studies at Oklahoma State University, Stillwater, Oklahoma in December, 2004.
Completed the requirements for a Bachelor of Science Degree in Food Technology at the University of Applied Sciences, Wädenswil, Zürich, Switzerland in February, 2002.

Experience:
Served as a teacher for home economics, from 1993 to 1998 in Basel, Switzerland. From 1999 to 2003 followed internships in different jobs in the food industry and related fields in Switzerland, Israel, and The Netherlands. Served as research and teaching assistant in the food and nutritional sciences fields at Oklahoma State University from 2003 to 2010.

Name: Maya Lucia Joray Date of Degree: July, 2011

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: EVALUATION OF NEW TECHNIQUES FOR ZINC AND CORTISOL ASSESSMENT WITH A PLACEBO-CONTROLLED ZINC SUPPLEMENTATION TRIAL IN A SUBSAMPLE OF ETHIOPIAN WOMEN

Pages in Study: 198 Candidate for the Degree of Doctor of Philosophy

Major Field: Nutritional Sciences

Scope and Method of Study:

The placebo-controlled zinc supplementation (20 mg zinc daily) study investigated the changes in plasma zinc levels, as well as other health and stress parameters at baseline and endpoint of the 17 day trial. In addition, a novel method quantifying changes in zinc transporter Zip 4 mRNA expression due to increased dietary zinc intake was tested. Additionally, the correlation between the newer salivary cortisol measurement to the established urine and serum cortisol measures was tested. The effect of changes in zinc intake on DNA integrity was evaluated employing the comet assay.

Findings and Conclusions:

No significant intervention effect on plasma zinc or other measured health or stress parameters could be identified. The volunteers were within normal ranges for the parameters assessed in this study, except for their moderate iodine deficiency. Due to the fact that not enough mRNA could be extracted from the samples, the developed method could not be tested with the samples obtained in the field. Saliva cortisol did correlate with the urinary cortisol levels; however, a quarter of the volunteers had saliva cortisol concentrations were slightly above normal range of the test kit, while all urine and serum samples were within normal range. The comet assay revealed that zinc supplementation significantly decreased DNA breaks.

ADVISER'S APPROVAL: Dr. Barbara J. Stoecker