

Chapter 13

Alcohol-Mediated Zinc Deficiency Within the Alveolar Space: A Potential Fundamental Mechanism Underlying Oxidative Stress and Cellular Dysfunction in the Alcoholic Lung

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Abstract Zinc is one of the most abundant trace elements in the human body, and its presence is essential for numerous biological processes including enzymatic activity, immune function, protein synthesis, and wound healing. Given these important roles, zinc has a sophisticated transport system to regulate its homeostasis. Determination of zinc status, however, is difficult to determine as serum levels are closely maintained and are not an accurate reflection of total body zinc or metabolism at the organ level. Fortunately, the discovery of zinc-specific fluorescent dyes has allowed for a much better assessment of zinc status in the respiratory system and has revealed that alcoholism perturbs this highly developed zinc metabolism such that its distribution to the lung and alveolar space is significantly decreased. As a result, this pulmonary zinc deficiency impairs function in the alveolar macrophage, which is the primary host immune cell within the lower airway. Experimental models have demonstrated that correction of this zinc deficiency restores immune function to the alveolar macrophage as best reflected by improved bacterial clearance in response to infection. While the precise mechanisms underlying alcohol-induced zinc deficiency are still under investigation, there is experimental evidence of several important connections with granulocyte–macrophage colony-stimulating factor and oxidative stress, suggesting that alteration of zinc homeostasis may be a fundamental mechanism underlying the cellular pathology

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seen in the alcohol lung phenotype. This chapter reviews zinc homeostasis and offers insight into our understanding of zinc deficiency in the setting of alcoholism and the potential of zinc as a therapeutic modality in the vulnerable alcoholic host.

Keywords Zinc • Glutathione • Alveolar macrophage • Pneumonia • Phagocytosis • Alcohol • Granulocyte/macrophage colony-stimulating factor (GM-CSF)

Basics of Zinc Homeostasis

Zinc is the second most abundant trace metal in the human body (behind iron), and it plays a crucial role in normal homeostasis within multiple organ systems. As it is one of 16 essential trace elements, humans require a relatively small amount of zinc, but it must be ingested from exogenous sources since the body cannot produce it endogenously. According to the Office of Dietary Supplements (ODS), the primary source of zinc for most individuals in the United States is protein-dense foods such as red meat and poultry. The food source with the most zinc is oysters, one serving of which has over ten times the zinc content as a serving of red meat [1]. However, other foods may be fortified with zinc, and it is also available as a dietary supplement and as an active ingredient in certain over-the-counter cold remedies. The Recommended Dietary Allowance (RDA) for zinc is 11 mg/day for adult males and 8 mg/day for adult females [2]. Only about 20 % of ingested zinc is actually absorbed into the bloodstream, and the rest remains in the gastrointestinal tract where it is excreted in the feces [3]. In the bloodstream, the form of zinc that is found in the plasma is one that is primarily bound to plasma proteins, mostly albumin.

Regulation of Zinc Transport and Storage

Zinc is important in various cellular processes. Most recognized is its role as a cofactor for the catalytic activity of over 300 enzymes [4] including multiple enzymes that are critically involved in antioxidant defenses. In parallel, zinc plays a key role in regulating gene expression as thousands of transcription factors are the so-called zinc finger proteins [5]. In fact, it has been estimated that approximately 10 % of the human genome encodes proteins that require zinc for their functional activity. Not surprisingly, due to its widespread effects zinc metabolism influences a wide array of important bodily functions including protein synthesis, wound healing, immune function, and DNA synthesis [6]. Given these far-reaching effects in the body, proper zinc homeostasis requires a sophisticated transport and regulatory system to ensure that it is readily available for the various cellular processes that require its presence.

There are two families of transmembrane zinc transport proteins that control its movement in and out of cells and subcellular compartments [7]. These are the Zip and Znt families of transporters, which have opposite or contrasting functions in

regulation of zinc homeostasis. The Zip family of transporters, of which there are 15 in humans, are zinc import proteins that function to increase the cellular concentration of zinc by its uptake from the extracellular space or by the release of zinc from intracellular vesicles. In contrast, there are nine known Znt transporters in human beings, and they function to decrease the cytoplasmic concentration of zinc by promoting its efflux from the cell or its influx into intracellular vesicles [8]. Studies have shown that the primary site of regulation for these transporters occurs in the intestine, where zinc is absorbed. For instance, in states of zinc deficiency, upregulation of Zip transporters occurs to ensure that more dietary zinc is absorbed and less is excreted in the feces [9]. In addition, metallothionein is a zinc storage protein and an indicator of overall cellular zinc status. Production of metallothionein is highly dependent on the availability of dietary minerals including zinc but is not specific to zinc alone.

The Role of Zinc in the Immune Response

In addition to its important role in enzymatic activity, zinc sufficiency is critical for normal immune function. Various studies have shown that zinc deficiency causes atrophy of the thymus, which results in lymphopenia and compromises overall T lymphocyte response and cell-mediated immunity [10]. Experimental models of zinc deprivation also result in defects of the bone marrow and affect the B-cell lineage as well [11]. Clinically, zinc deficiency has recently been shown to play an important role in patients with immune system disorders such as common variable immunodeficiency (CVID) [12]. While depletion of zinc in these patients does not actually cause the immune syndrome, its deficiency can certainly exacerbate the already impaired host response. Comparable to its role in CVID, zinc deficiency has been reported in both patients with human immunodeficiency virus (HIV) [13] as well as in experimental models of HIV infection [14, 15]. In addition to these observed derangements in acquired immunity, zinc deficiency has been shown to cause various deficits in innate immune function as well. Specifically, studies have shown that zinc depletion results in dysfunction of macrophages [16, 17]. This is of particular consequence in the respiratory system where alveolar macrophages are constantly exposed to environmental pathogens and debris and are the first line of defense against various pulmonary infections including pneumonia. Further evidence for the role of zinc in the immune response may be derived from studies of people with the prototypical zinc metabolism disorder, acrodermatitis enteropathica, which is a rare genetic disease characterized by an inability to uptake zinc due to the lack of an important zinc transport protein. Patients with acrodermatitis enteropathica have been used to describe the hallmarks of severe zinc deficiency, and these individuals demonstrate several important defects in host defense [18].

The burden of nutritional zinc deficiency is much more evident in Third World countries where poverty-stricken individuals have limited access to necessary food sources required to formulate a well-balanced diet. This disposition is especially

apparent among young children. Studies show that children in several African countries are much more prone to the development of pneumonia, and this deficiency has been linked specifically to zinc deficiency [19, 20]. International health initiatives have led to the development of organized campaigns to advertise the effectiveness of dietary zinc supplementation in areas where children are most vulnerable. Similarly, in the United States, inadequate dietary zinc intake has been described in children living in families of low socioeconomic status, in infants with low birth weight, and among teenagers who become pregnant [10, 21]. Interesting experimental models have verified the key role of zinc deficiency specifically in pulmonary immune function. In these experiments, researchers zinc-deprived a group of mice and immunized them to pneumococcus, a common cause of bacterial pneumonia. After allowing for immunity to develop, they inoculated the mice with the pneumococcal organism into the lung and subsequently measured pneumonia severity. They demonstrated that pneumonia is much more severe in mice that are zinc-deprived and further that immunization is ineffective at preventing infection in the setting of zinc deficiency [22, 23]. In other words, zinc-deprived mice are not only unable to acquire immunity in response to vaccination, but they also develop a much more severe infection compared to mice that were not zinc-deprived. Taken together these studies illustrate that zinc is not only intimately connected with the global immune response but also crucial for immunity specifically in the pulmonary system.

Determination of Zinc Status

Currently, the only clinically available test to diagnose zinc deficiency is a serum zinc level. This measurement is performed by numerous commercial laboratories and can be accomplished with a simple blood test. While this is a straightforward diagnostic evaluation, the utility of the result has been questioned since plasma zinc only accounts for about 0.1 % of total zinc stores. Thus, serum measurements of zinc are an unreliable indicator of total body zinc, and blood levels of zinc often do not appreciably drop until zinc deficiency reaches a critical level. Further, various biological conditions and stressors can affect serum zinc levels. Studies show that tissue levels of zinc may be decreased even when serum levels are normal. Specifically, a recent study among alcoholic subjects revealed that alcoholics had evidence of zinc deficiency in the lung, but all had serum zinc measurements that were within the normal range. Further, serum zinc levels may be decreased in states of inflammation even in states of zinc sufficiency [24]. While the mechanisms for these findings are not entirely clear, studies have shown that inflammatory cytokines can upregulate the Zip family of transporters and may explain the transient decrease in serum zinc levels that has been observed with the acute-phase response [25]. Therefore, zinc-deficient states may exist with normal serum zinc levels, and plasma zinc deficiency can occur when total body zinc stores are normal. Given the inherent unreliability of serum zinc as a surrogate marker of zinc status, alternate methods have been developed to better assess zinc deficiency in the research setting.

Various zinc-specific dyes have been developed to evaluate zinc in different cell types and biological fluids. These dyes typically come in two forms: a membrane-impermeable form that is able to measure zinc in extracellular fluids and a membrane-permeable form to assess intracellular zinc levels. One particular dye, FluoZin-3, is manufactured by Invitrogen and has been used in various recent studies evaluating pulmonary zinc status. FluoZin-3 is a membrane-impermeable fluorochrome with a very high affinity for zinc but a low affinity for other cations such as magnesium and calcium. Therefore, it is an ideal way to measure extracellular zinc levels in biological specimens such as lung lavage fluid. The membrane-permeable form, FluoZin-3AM, has similar zinc-binding properties and has been used to measure intracellular zinc levels in different pulmonary cell types including alveolar epithelial cells and alveolar macrophages. Utilization of such methods has allowed for a much greater and more precise understanding of zinc metabolism in the respiratory system than evaluating serum zinc levels alone.

Zinc Metabolism in the Respiratory System

Given the clinical observations that zinc deficiency leads to an increased susceptibility to pneumonia and other pulmonary infections, groups of researchers began to investigate zinc metabolism at the organ level. Using the zinc fluorophore Zinquin, groups led by Truong-Tran [26] and Carter [27] were the first to image pools of zinc in respiratory cells. They were able to demonstrate the presence of zinc in the apical region of ciliated airway epithelial cells as well as in alveolar epithelial cells. In these same regions, there were important zinc-dependent enzymes such as caspase-3 and Cu/Zn superoxide dismutase. Zinc inhibits caspase-3, an enzyme that activates apoptosis, and superoxide dismutase has very important antioxidant function within the lung. Further, these researchers demonstrated that pools of zinc in these cell types were strongly decreased by treatment with zinc chelators, and there was a parallel increase in apoptosis and oxidative stress. Subsequent researchers have demonstrated that several important zinc transporters are also localized to the lung [7, 28] and are involved in regulation of zinc metabolism within the respiratory system. Taken together, these early studies highlight the presence and importance of zinc in the respiratory system.

The Effect of Alcohol Exposure on Overall Zinc Homeostasis

The presence of zinc deficiency among alcohol abusers with hepatic dysfunction has been recognized for over 50 years [29]. Studies show that individuals with alcoholic cirrhosis (as well as cirrhosis from other etiologies) have both serum zinc deficiency as well as decreased tissue levels of zinc [30]. However, much less is known about zinc levels in the alcoholic patient who has not developed liver disease, but studies

suggest that there are alterations in zinc metabolism [31, 32]. The mechanisms responsible for creating zinc deficiency in the alcoholic subject are not entirely clear. While the majority of Americans have no difficulty meeting the RDA for zinc, studies have shown that the vast majority of alcoholics are not able to meet this goal [32]. This is not surprising when taken in the context of the generally poor dietary habits that exist in this population. However, even with sufficient intake, experimental studies have demonstrated that the absorption of zinc is impaired by chronic alcohol ingestion [33], and there is also an increase in urinary zinc excretion among alcoholics [34]. An experimental model evaluated different zinc transporters in the small intestine in order to better understand the effects of zinc absorption in the setting of chronic alcohol ingestion [17]. While zinc deficiency would normally increase expression of Zip proteins to increase intestinal absorption of dietary zinc, this study demonstrated that alcohol exposure directly decreased Zip4 expression in the small intestine, thereby decreasing zinc absorption even in a state of zinc deficiency. Finally, one group of investigators showed that albumin has a much lower affinity for zinc in subjects with cirrhosis [35], a finding that may additionally contribute to the observed zinc deficiency in this population. While our overall understanding of the mechanisms involved in creating zinc deficiency among alcoholics is limited, it is likely that multiple mechanisms act in concert.

The Effect of Alcohol Exposure on Zinc Homeostasis in the Lung

In addition to its widespread medical consequences in other organ systems, chronic alcohol exposure causes various derangements in the respiratory system. Clinically, patients who abuse alcohol have a predisposition towards the development of pulmonary infections. The key factors associated with alcoholism that contribute to this increased susceptibility include a change in oral bacterial flora, diminished gag and cough reflexes that occur during inebriation, dysfunction of the cilia in the airway that assist with secretion clearance, and impairment of the alveolar macrophage, which is the primary immune cell in the lower respiratory system. Given the overwhelming evidence that zinc metabolism is crucial for the immune response and the significant findings that illustrate that chronic alcohol exposure alters zinc homeostasis, it is a logical progression to implicate zinc deficiency as a primary contributor to immune dysfunction and susceptibility to pulmonary infection in this vulnerable population.

Experimental models of alcoholism have established that extracellular zinc in lung lavage samples is decreased about 30 % in animals on an alcohol-fed diet compared to control-fed diet. In these models, chronic alcohol exposure directly causes alterations in zinc metabolism since the diets are otherwise similar from a caloric standpoint and overall zinc content. This is an important finding since studies of human subjects implicate poor nutritional status as an important cause of zinc and other nutritional deficiencies that accompany alcoholism. These findings contend

that merely “recommended” nutritional intake may not be enough to combat zinc deficiency with alcohol abuse. In this same experimental model, dietary zinc supplementation is able to reverse zinc deficiency in the alveolar space and improve alveolar macrophage immune function. The recommendations for zinc replacement for individuals with documented zinc deficiency are about five to ten times greater than the RDA for zinc, which is comparable on a milligram per kilogram basis to the dose of zinc supplementation used in this animal model of chronic alcohol ingestion. More recent evidence in human subjects has shown that alcoholic subjects have a similar 30 % decrease of intracellular zinc in alveolar macrophages compared to matched nonalcoholic subjects, validating the findings in experimental models of chronic alcohol ingestion [24].

In addition to alcohol-mediated decreased zinc levels in the alveolar space, there is also alteration of zinc transporter metabolism. It is expected that zinc import proteins, or Zip transporters, would be upregulated in the setting of zinc deficiency. However, alcohol decreases several important Zip proteins in alveolar macrophages, including Zip1 and Zip4 [17]. These findings demonstrate that alveolar macrophages are further impaired in their ability to uptake zinc during this state of global zinc deficiency. This is parallel to the effect of alcohol on decreasing important zinc transporters in the small intestine where zinc is absorbed from the diet and illustrates that alcohol has direct effects in altering zinc metabolism in multiple organ systems including the lung.

While zinc levels are clearly decreased in experimental alcohol-fed animals, these models have further demonstrated that zinc deficiency in the setting of chronic alcohol ingestion contributes to both alveolar epithelial barrier dysfunction and alveolar macrophage immune impairment. These novel investigations argue that zinc deficiency may be a unifying mechanism for the alcohol lung phenotype and explain alcohol-induced susceptibility to pneumonia and acute lung injury.

Effect of Zinc Deficiency on Alveolar Epithelial Barrier Function

Clinically, alcoholics have greater than twofold increase in the risk of developing acute lung injury and acute respiratory distress syndrome (ARDS), and when these devastating conditions do occur alcohol abusers have a higher severity of illness and greater mortality than their nonalcoholic counterparts [36–38]. While the exact mechanisms for these findings are still under investigation, studies have shown that alcohol abuse impairs alveolar epithelial barrier function, resulting in greater fluid leak and an increased propensity to develop lung edema [39–42]. Granulocyte–macrophage colony-stimulating factor (GM-CSF) has been implicated, at least partly, in alcohol-induced epithelial barrier dysfunction. GM-CSF is a 23-kDa peptide that is secreted by several cell types, including alveolar epithelium, and has many important functions in the lung. Chronic alcohol ingestion has been shown to decrease GM-CSF receptor expression and signaling in the

alveolar epithelium, causing impairment in GM-CSF-dependent epithelial barrier formation [43]. Importantly, in experimental models of chronic alcohol ingestion, recombinant GM-CSF treatment is able to normalize epithelial barrier function [44] and improve expression of tight junction proteins that are altered by alcohol exposure [45]. More recently, it was determined that treatment of zinc deficiency with supplementation *in vitro* and *in vivo* improves GM-CSF receptor expression and reverses alveolar epithelial barrier dysfunction [17]. Specifically, epithelial barrier integrity was assessed by measuring paracellular leak of radiolabeled sucrose in established monolayers of epithelial cells. These experiments were performed in both alcohol-treated alveolar epithelial cell lines and cells that were isolated from alcohol-fed animals, and zinc supplementation was performed *in vitro* for cell lines and added to the diet of alcohol-fed animals. Further, in this same model, dietary zinc supplementation increased gene and protein expression of important tight junction proteins that are affected by chronic alcohol exposure. While the precise connection between zinc deficiency and GM-CSF signaling remains under exploration, these novel findings argue that zinc deficiency may be a fundamental mechanism in alcohol-induced epithelial barrier dysfunction and better explain the predisposition alcoholics have to the development of acute lung injury and ARDS.

Effect of Zinc Deficiency on Alveolar Macrophage Function

Pneumonia and aspiration are the most common pulmonary risk factors for the development of acute lung injury and ARDS, and alcoholics have an increased susceptibility to both conditions. The risk of aspiration is primarily related to diminished consciousness associated with intoxication, and the risk for pneumonia (and other pulmonary infections) is the result of weakened host immunity. The alveolar macrophage is the primary immune cell in the lower airways and the first line of defense against invading pathogens. Several experimental studies have demonstrated that chronic alcohol ingestion impairs alveolar macrophage immune function [46–48]. Just as GM-CSF is important for epithelial barrier function, its signaling is crucial for alveolar macrophage differentiation, maturation, and function. GM-CSF signaling occurs through receptors on the cell surface of numerous cell types, including alveolar macrophages. In parallel to its effects on the alveolar epithelium, chronic alcohol exposure decreases expression of GM-CSF receptors and signaling through its master transcription factor PU.1 in alveolar macrophages [48]. Further, recent evidence has shown that treatment of alcohol-induced zinc deficiency with dietary zinc supplementation improves GM-CSF receptor expression and restores alveolar macrophage immune function [17]. In this particular experimental model, dietary zinc supplementation increases expression of both the GM-CSF α binding subunit and the GM-CSF β signaling subunit. These findings add to a growing body of evidence implicating zinc deficiency as an important mediator of immune dysregulation in the alcoholic lung.

Role of Oxidative Stress

Early observations of the alcohol lung phenotype consistently demonstrated an increase in oxidative stress in the lower airways caused by alcohol abuse [49–51]. Oxidative stress had previously been demonstrated in other tissues affected by alcohol abuse such as the liver [52]. More specifically, extensive research has implicated alcohol-mediated depletion of glutathione (GSH) as a primary driver of oxidative stress in the liver [53] and respiratory system [51]. GSH is a tripeptide that is generated primarily by the liver and is employed in various detoxification pathways and oxidant-mediated cytokine generation [54]. While the exact mechanism has yet to be elucidated, studies have shown that alcoholism decreases GSH levels in the liver even when cirrhosis is not present [53], and treatment with GSH precursors restores oxidant balance and prevents the development of alcohol-induced liver injury in experimental models [55]. GSH is an important cellular antioxidant in the alveolar space, and GSH precursors have a similar therapeutic value in reversing alcohol-mediated pulmonary defects associated with redox stress [50, 56–58].

Oxidative stress is the result of disparity in production and neutralization of reactive oxygen species (ROS), which occurs when there is an imbalance in the oxidized and reduced forms of a redox pair. Synthesis of GSH requires cysteine (Cys), and recent experimental evidence has suggested that Cys and its oxidized counterpart cysteine (Cyss) operate at the extracellular level and GSH and its oxidized form glutathione disulfide (GSSG) function as an important intracellular redox pair [59]. Redox pairs are one means of combating oxidative stress, but there are other important defenses as well. For instance, the antioxidant response element (ARE) is a genetic program that is triggered in the setting of oxidative stress. ARE signaling occurs through its master transcription factor Nrf2, and the ARE/Nrf2 pathway is a more diverse and comprehensive host defense mechanism against redox stress. Studies have suggested that the ARE is an important means of host protection against oxidative stress specifically in alcoholism [60–62].

Appropriate zinc metabolism is vital for the production and activity of numerous antioxidants, and zinc deficiency alone impairs host defense against redox stress [4]. Indeed, zinc deficiency in settings independent of alcoholism has been shown to induce a state of oxidative stress in the respiratory system [63, 64]. More recently, alcohol-induced zinc deficiency has been shown to increase oxidative stress in the lungs and decrease signaling through Nrf2 [65], illustrating a novel mechanism by which alcoholism may exacerbate oxidative stress in the lower airways. More importantly, zinc supplementation was able to restore redox balance and improve Nrf2 activity in these experimental models. Further, specialized assays illustrate that Nrf2 and PU.1 cooperatively bind to DNA, thereby providing important experimental evidence that the GM-CSF and ARE pathways are linked in a zinc-dependent fashion. These observations imply that zinc deficiency is a fundamental mechanism by which alcohol abuse induces oxidative stress and cellular dysfunction within the alveolar space. Further, these experimental models have provided a strong basis to begin exploring the potential therapeutic role of dietary zinc supplements in a vulnerable alcoholic population.

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