THE ROLE OF NUTRITION
DURING THE EARLY INFLAMMATORY STAGE
OF CUTANEOUS WOUND HEALING

DISSERTATION

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* * * * *

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ABSTRACT

Although various factors are involved in the wound healing process, malnutrition may be a major factor that can lead to tissue damage and delay wound healing. It is well known that malnutrition triggers compromised immune functions and decreased antioxidant defense in many studies. Moreover, immune response and antioxidant defense are critical facets of the inflammatory stage that can be the rate-limiting step of later stages of wound healing. Thus, nutritional modulation of immune function and antioxidant status may play a crucial role in the control and regulation of wound healing.

Reactive oxygen species (ROS) and proinflammatory cytokines produced by immune cells during inflammation activate NFκB, a redox sensitive transcription factor that induces expression of immunoregulatory genes such as chemokines and cytokines during the inflammatory stage. NFκB activation and proinflammatory cytokine production play key roles in regulating wound healing. Notably, protein energy malnutrition (PEM) and zinc deficiency are well-known health problems associated with delayed wound healing. N-acetyl cysteine (NAC) supplementation in PEM may help wound healing by enhancing immune response and antioxidant defense. Zinc supplementation may also increase immune function and antioxidant defense. Therefore, this dissertation was focused upon the role of nutrition and ROS in wound closure and the effect of nutritional supplementation on immune response and antioxidant defense at
cellular and molecular levels during the early inflammatory stage of cutaneous wound healing.

We have hypothesized that malnutrition delays cutaneous wound closure due to decreased immune response and antioxidant defense. Furthermore, we propose that nutritional supplementation will restore immune function and antioxidant defense in the inflammatory stage during cutaneous wound healing. To test these hypotheses, we have: i) investigated the effects of PEM and the role of ROS using CuZnSOD transgenic mice to determine if malnutrition or ROS affect cutaneous wound healing; ii) examined the role of dietary supplementation of NAC on wound closure and gene expression of pro-inflammatory cytokines (IL-1β and TNF-α) and IκB (indirect measurement of NFκB activation) during the early inflammatory stage in PEM mice; and iii) investigated the role of dietary zinc on wound closure and gene expression of IL-1β, TNF-α and IκB.

The results of these experiments demonstrated that PEM impaired wound healing, possibly due to delayed neutrophil infiltration and decreased gene expression of IκB, IL-1β and TNF-α. However, NAC supplementation restored neutrophil response and normalized gene expression of IκB, IL-1β and TNF-α in the early inflammatory stage of cutaneous wound healing. In addition, we found that zinc deficiency delayed wound closure. Notably, we also found that zinc supplementation at 500 ppm accelerated neutrophil infiltration, increased expression of IκB and enhanced wound closure. However, mega dose zinc supplementation at 1000 ppm (20 times higher than that of a control diet) delayed neutrophil infiltration, decreased IκB levels and delayed normal wound closure. In conclusion, this study provides further evidence of the critical role of nutrition in wound healing. More importantly, results from these experiments
demonstrated that NAC supplementation provides an effective intervention strategy to enhance wound healing in PEM patients. However, we have also demonstrated that supplementation strategies must be approached judiciously, as our results show that whereas zinc supplementation at 500 ppm may enhance wound healing, zinc supplementation at 1000 ppm significantly delays wound healing.
DEDICATION

This dissertation is dedicated with love to my family for their love and support through the years.
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Lastly, I would like to thank my family for all their love and support, which helped get through the years and have the ‘Ph.D.’. I will return this to my parents.
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PUBLICATIONS

Research Publication

1. Hyun-Sook Kim, Yunsook Lim, Chun-Sik Park, Modulation of Cytokine
   Production by Nutritional Status in Elderly Women, The Korean Journal of

2. Yunsook Lim, Hyun-Sook Kim, Chun-Sik Park, Comparative Analysis of
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<td>CAT</td>
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<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
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<td>GSH</td>
<td>Glutathione</td>
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<td>GSH</td>
<td>Glutathione-S-transferase</td>
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<td>IkB</td>
<td>Inhibitory kappa B</td>
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<td>IL</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>NAC</td>
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<td>NFκB</td>
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<td>PEM</td>
<td>Protein energy malnutrition</td>
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<td>Reactive oxygen species</td>
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<td>Superoxide dismutase</td>
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<td>TNF-α</td>
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CHAPTER 1

INTRODUCTION

Wound healing is an essential process that serves to repair and regenerate tissue structure and function that has been disrupted, or wounded, by physical, chemical, bacterial or viral insults. In general, there are four major stages of wound healing: clot formation, inflammation, proliferation and remodeling (Figure 1.1). However, wound healing is in fact a complicated cascade of processes involving the coordinated interaction of inflammatory cells, fibroblasts, keratinocytes, endothelial cells, epithelial cells, chemotactic factors, cytokines, growth factors, neurotransmitters, extracellular matrix proteins, proteinases, hormones, reactive oxygen species (ROS) and nutrients (1-3). Indeed, the coordinated interaction of both cellular and acellular components of the wound healing process is critical, as modulation of these factors may exacerbate tissue inflammation and trigger the onset of more severe disease. In fact, chronic wounds and their treatment pose a serious problem to the healthcare system and may cost $5 billion to $9 billion annually (4). The role of the immune system in wound healing has been reviewed by others (5-8). Thus, this review will focus on the effect of nutrition and the role of ROS in the early inflammatory stage that modulates entire wound healing process.
Inflammation and Wound Healing

Molecular and cellular interactions in each stage of wound healing have been well investigated. The wound healing process is a cascade of processes and each stage of wound healing, clot formation, inflammation, proliferation and remodeling, is temporally overlapped (Figure 1.1)(9). Therefore, because the inflammatory stage begins immediately after the wound has been inflicted, it may be the most important stage in terms of regulation of entire healing process, particularly with regard to the role of proinflammatory cytokines and the gene transcription factor, nuclear factor kappa B (NFκB).

During the early inflammatory stage, several inflammatory cells, predominantly neutrophils, migrate to the wound site followed by macrophages and later lymphocytes. These cells are recruited by chemokines and chemotactic factors such as interleukine (IL)-8, growth related oncogene (GRO)-α, and monocyte chemotactic factor (MCP)-1 (10). Neutrophils normally arrive at the wound site within minutes to clear contaminating bacteria and the number of neutrophils typically peaks at 24 hour after wounding. Neutrophils produce proinflammatory cytokine including IL-1β and tumor necrosis factor (TNF)-α (9). Macrophages follow neutrophils into the wound site and continue clearing cellular debris including neutrophils through phagocytic processes that involve the production of ROS including superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) (5). Macrophages also secrete growth factors such as platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and transforming growth factor (TGF)-β as well as proinflammatory cytokines (11-13). Proinflammatory cytokines, especially IL-1β and TNF-α and growth factors also serve to recruit more inflammatory...
cells, enhance phagocytosis and stimulate fibroblast proliferation and keratinocyte
growth, the synthesis and degradation of extracellular matrix proteins, angiogenesis and
fibroblast chemotaxis during proliferative stage (8,13-15). Furthermore,
proinflammatory cytokines play crucial roles in activating immunoregulatory genes such
as chemotactic factors, cytokines, cell adhesion molecules and growth factors regulated
by a redox-sensitive gene transcription factor, NFκB through feedback and feed-forward
amplification (16-20). Thus, dysregulated production of proinflammatory cytokines and
NFκB may cause delayed or impaired wound healing (16,17).

**ROS and Wound Healing**

Oxygen is essential in collagen synthesis, epithelialization, and wound infection
but the role of oxygen metabolites, particularly ROS, is controversial with respect to
wound healing (21). During the respiratory burst, inflammatory cells such as neutrophils
and macrophages produce superoxide (O$_2^-$) from oxygen using NADPH cytochrome b
oxidase (22,23). Neutrophils produce ROS and hydrogen peroxide (H$_2$O$_2$) to kill
bacteria, to prevent infection and to induce vascular endothelial growth factor (VEGF)
production by macrophages (24). Hydrogen peroxide may also suppress epithelial cell
migration and proliferation at this early stage of wound healing (25,26). Pro-
inflammatory cytokines stimulate the production of ROS in fibroblasts for wound
granulation (27). Nitric oxide (NO) produced by macrophages plays beneficial roles in
collagen deposition and increased tensile strength in wound healing (28). Thus, in
addition to their central role in mediating bacterial and viral death, ROS produced by
inflammatory cells are also beneficial in recruiting other inflammatory cells, cytokine
production and modulating collagen synthesis during the wound healing process. Furthermore, recent evidence indicates that low-level ROS may serve as signaling molecules and induce the expression of genes involved in the wound healing process (29,30). However, excessive ROS production can cause tissue damage to induce chronic disease status such as cancer and atherosclerosis (21,31-33). The importance of ROS has been investigated indirectly through antioxidant defense system, non-enzymatically and enzymatically (34,35). SOD mRNA is significantly induced in the early stage of wound healing (5). In addition, the levels of antioxidant enzymes and nutrients such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-S-transferase (GST), vitamin C and vitamin E decrease with delayed wound healing (36,37). Therefore, treatments of antioxidants, SOD and allopurinol improve breaking strength and hydroxyproline content in skin wound (21). In many studies, antioxidant defense mechanism protects tissue damage from excessive ROS but it is not clear the role of ROS and antioxidant defense system in malnourished subjects in wound healing.

**Proinflammatory Cytokines, ROS and NFκB in Wound Healing**

During the inflammatory stage, proinflammatory cytokine production is initially induced by the alternative complement pathway, which is activated by bacteria and foreign material (38,39). For example, TNF-α, a primary proinflammatory cytokine functions to activate leukocytes and in particular plays a role in neutrophil superoxide production, cytotoxicity and phagocytosis (40-44). Synthesis of the cytokine, IL-1β is also induced by the complement pathway and is itself an activator of neutrophils. It also functions as a chemotactic factor of fibroblasts for granulation and as a regulator of
collagen synthesis, methalloproteinase activity and the production of cytokines such as IL-6 and IL-8. In addition, both TNF-α and IL-1β stimulate NFκB activation and ROS production in inflammatory cells (15,17,27).

NFκB has pleiotropic functions in immunity. NFκB is normally an inactive form in the cytoplasm, bound to the inhibitory protein, IκB. During activation by various stimuli such as UV, ROS, proinflammatory cytokines and virus, NFκB is detached from phosphorylated IκB and is translocated to the nucleus to activate the expression of immune and inflammatory genes (45). Proinflammatory cytokines as stimulators of NFκB activate the expression of immunoregulatory genes including chemotactic factors, cytokines, adhesion molecules and growth factors regulated by NFκB through feedback and feed-forward amplification (16-20).

**Nutrition and Wound Healing**

Nutritional status has been shown to modulate antioxidant/oxidant balance by changing the redox state of cells either enhancing the ROS production or altering antioxidant defenses as well as immune function by altering the membrane composition of immune cells which produce and response to proinflammatory cytokines after injury (46). In addition, because wound healing is an anabolic-energy requiring process the balanced nutrition is important to provide substrates for the synthesis of proteins and peptides to support the enhanced metabolic flows and cellular synthesis during inflammation and immune responses proper wound healing (46-48). Therefore, nutrition is a critical factor in the wound healing process.
In previous studies, deficiencies of macro- and micronutrients including protein, amino acids, vitamins and minerals result in impaired healing process by changes in various factors involving wound repair and regeneration (49) (reviewed by (50,51)). For examples, vitamin A or vitamin C deficiency delays wound healing and increases susceptibility to infection (52,53). Nutritional supplementation is still controversial to enhance wound healing. Arginine supplementation has increased collagen deposition (54,55). However, supplementation of vitamins such as vitamin C has not shown to enhance wound healing in healthy subjects (56,57). Because most nutritional supplementation studies have been conducted in healthy subjects, the effect of nutrition on wound healing in malnourished subjects is still unknown. Therefore, this review will focus on the effect of protein energy malnutrition (PEM) and zinc deficiency on wound healing in immune aspects among many nutritional deficiencies.

i) PEM, Immunity and Wound Healing

Protein energy malnutrition (PEM) is a well-known health problem associated with high mortality and morbidity in developing countries. In affluent countries, PEM secondary to chronic disease such as cancer, AIDS, and gastrointestinal disorders is a prevalent crisis in adults (58). Notably, PEM is characterized by impaired immune function which may contribute to high morbidity and mortality (59). For example, the infiltration of neutrophils and macrophages into injured tissues is impaired and the proliferation, differentiation and the release of leukocytes from the bone marrow are decreased in PEM animals (60). PEM may also impair phagocytic function, ROS and cytokine production (61-64). For example, levels of TNF-α and IL-6 in blood are
reduced in PEM children but IL-6 concentration is increased in post-operative PEM patients (65,66). The production of IL-1, IL-2 and IFN-γ and the production and release of lysozyme are also decreased in PEM subjects (67).

In PEM patients, levels of antioxidants such as vitamin C, vitamin E, glutathione (GSH), GPx are reduced and free radical production is increased (68,69). Decreased immune response, increased ROS production and an impaired antioxidant defense system in PEM subjects may increase their susceptibility to infection after wounding. Indeed, delayed wound healing in post-operative PEM patients is a known predicament. In acute malnutrition, the hydroxyproline content and collagen expression are decreased at the wound site. The concentrations of nitrite/nitrate and citrulline, substrates of NO, in wound fluid and wound cell supernatants are deceased in PEM (28). PEM also causes decreased granulation tissue formation and extracellular matrix molecules and delayed wound healing (70-75). In addition, the decreased number and function of leukocytes in PEM patients may prolong bacterial residency time and decreased production of inflammatory cytokines at the wound site.

**NAC Supplementation in PEM**

PEM is related to an imbalance between ROS production and antioxidant defense and impaired immune function (68). To enhance antioxidant defense and immune function proper treatment is required during the rehabilitation period for PEM. However, a high protein and energy rich diet is not recommended to PEM patients during initial stage of rehabilitation because this diet may cause toxicity due to catabolic changes of body metabolism in PEM subjects (76). Therefore, therapeutic strategies for PEM
patients designed to increase immune function and antioxidant defenses without high 
protein and energy rich diet may be critical during the early stages of rehabilitation. 
Notably, many studies show that the level of the tripeptide glutathione (L-γ-glutamyl-
cysteinyl-glycine, GSH) is decreased in tissues such as liver and lung of PEM patients 
and animals and it is associated with impaired immune function and increased 
susceptibility to infection (77-80). GSH takes part in detoxification of drugs, 
maintenance of intracellular protein integrity, regulation of signal transduction pathway 
and the increase in immune defense (81-86).

Cysteine is the limiting amino acid in GSH synthesis but cysteine itself cannot be 
given to increase tissue GSH levels because of its toxicity at high concentration (68). 
However, N-acetyl cysteine (NAC), a cysteine prodrug, is a well known bioavailable 
cysteine precursor that is rapidly converted into cysteine and GSH in liver (85). 
Moreover, decreased immunity due to low intracellular GSH is restored by NAC 
supplementation in disease states such as cancer and AIDS (87).

NFκB activation is decreased in macrophages of PEM mice but NFκB activation 
induced by LPS in PEM mice is increased and enhanced with a structural modification of 
an active form of NFκB and NAC supplementation normalizes NFκB activation in PEM 
mice (88,89).

ii) Zinc, Immunity and Wound Healing

Zinc deficiency often accompanied with PEM is a health problem in United States 
and is associated with chronic diseases such as cancer, renal diseases, alcoholism and 
gastrointestinal disorders. Zinc deficiency is characterized by growth retardation, high
susceptibility to infection, cognitive impairment and poor wound healing (90). Zinc deficiency is also associated with impaired immune function. Cell mediated immunity such as T and B lymphocyte functions, neutrophil and NK cell activities, is impaired in zinc deficiency. In addition, the production of cytokines, including inflammatory cytokines, is reduced in zinc deficient subjects (91,92). However, supplementation with megadoses of zinc (more than 20 times of RDA) results in adverse effects in both innate and acquired immune function possibly associated with copper deficiency (93-95).

The role of zinc in the wound healing process has been investigated since the 1950’s (96). Zinc as a component of metalloenzymes as well as matrix enzymes is involved in promoting cell proliferation and reepithelialization (97). Zinc deficiency has been reported to increase time for wound closure and to decrease wound strength (98,99). Zinc has been used as an agent to treat diaper rash and zinc supplementation has been conducted in the patients with bedsores, ulcers, and incisional wounds. Several studies show that serum zinc concentration is decreased but zinc level in wound edge is increased during the proliferative stage (97,98,100). Topical treatments of zinc oxide in leg ulcers and skin wounds promote wound healing in some studies but the results are still controversial (101-105). Use of zinc tape on wound is beneficial in wound closure and collagen synthesis (106,107). Zinc treatment increases cytotoxicity of neutrophils by reducing toxin-producing bacteria (108). However, high serum zinc concentrations may inhibit proper healing and impair phagocytosis and high dose of zinc supplementation over 150mg/day may inhibit copper metabolism (109-111). The previous studies suggest that optimal zinc supplementation play beneficial roles in enhancing antioxidant defense and immune defense in wound healing process.
It has been known that ROS production is tightly regulated by enzymatic and non-enzymatic antioxidant systems and immune functions, which are mostly regulated by nutritional status, for decades. Therefore, a balanced diet maybe a key factor to regulate proper repair and regeneration in wound healing. Most nutritional research has focused on phenomena during the later stages such as hydroxyproline content, collagen deposition and tensile strength during wound healing and nutritional supplementation has not conducted in sick and malnourished wounded patients. Furthermore, the roles of nutrients at cellular and molecular levels are still unknown during the inflammatory stage, which is a regulating step of wound healing process. It is possible that nutritional supplementation in clinical nutritional problems may help to alleviate more tissue damage and accelerate proper healing. This research goal is to understand effects of malnutrition and roles of nutrient supplementation during the inflammatory stage, a controlling step, at cellular and molecular levels in cutaneous wound healing. We hypothesize that wound healing will be delayed in malnutrition including PEM and zinc deficiency possibly due to compromised immune function and antioxidant defense and will be normalized with nutrient supplementation by enhancing immune function and antioxidant defense. Specifically, we focus on inflammatory cell infiltration during the early inflammatory stage of wound healing because inflammatory cells play important roles in phagocytosis, productions of cytokines and chemokines for cascade process of wound healing. Furthermore, we focus on the gene expression of proinflammatory cytokines, IL-1β and TNF-α, which activate NFκB. Inappropriate activation of NFκB induces improper immune response and impaired wound healing,
We use an excisional skin wound model made by a biopsy punch which is well characterized healing process similar to human wound healing and is convenient to analyze wound closure and to investigate cellular and molecular events during healing process (112). Furthermore, our laboratory uses CuZnSOD transgenic (overexpresser or knockout) mice to examine the role of ROS and CuZnSOD during cutaneous wound healing.

Therefore, this dissertation focuses on i) the effects of PEM and the role of ROS using CuZnSOD transgenic mice on wound closure and immune response during cutaneous wound healing ii) the roles of dietary supplementation of NAC in the early inflammatory stage by examining infiltration of the inflammatory cells, the expression of proinflammatory cytokines, IL-1β and TNF-α and inhibitory kappa B (IκB), during cutaneous wound healing in PEM mice iii) dietary effects of zinc on descriptive wound closure, infiltration of the inflammatory cells, the expression of IL-1β and TNF-α and IκB during the early inflammatory stage of cutaneous wound healing.

This research will help to develop an understanding of the mechanism of gene expression of proinflammatory cytokines modulated by NFκB during cutaneous wound healing. In addition, the result of the research will be used for accelerating healing process and eventually decrease mortality and morbidity in various diseases caused by tissue damage through nutritional supplementation and reduce health costs.
Figure 1.1 Four different stages of wound healing
1.1 REFERENCES


CHAPTER 2

NUTRITION AND ROS AFFECT WOUND CLOSURE DURING CUTANEOUS WOUND HEALING

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2.1 SUMMARY

Nutrition and reactive oxygen species (ROS) are associated with immune function and antioxidant defense. However, the effect of nutrition and the role of ROS in wound healing process are still unclear. In these experiments we have hypothesized that PEM will increase the time of wound closure and decrease inflammatory cell infiltration into the wound site during the early inflammatory stage. In addition, because of the critical importance of ROS as mediators of cell signaling and phagocytic processes during wound healing, we have hypothesized that cutaneous wound healing will proceed more quickly in CuZnSOD knockout (CuZnSOD\(^{-/-}\)) mice compared to CuZnSOD wild-type (CuZnSOD\(^{+/+}\)) mice.

Weanling CD1 mice were fed a protein deficient diet (a 0.5% protein diet) for 2 weeks to investigate the effect of PEM. CuZnSOD transgenic (over-expresser, wild-type and knockout) mice were fed a control diet or a protein deficient diet for 2 weeks to examine the role of ROS on wound closure in cutaneous wound healing. Full thickness excisional wounds were made after diet treatment and wound closure was measured daily in each group. Neutrophil infiltration was examined by hematoxyline and eosin (H&E) staining in PEM mice. Wound closure and neutrophil infiltration were delayed in PEM mice when compared to those of control group and pair fed group. Neutrophil
infiltration and wound closure were accelerated in pair fed group when compared to those of control group. In the second study, wound closure was slower in CuZnSOD over-expresser mice fed a control diet but was slower in CuZnSOD knockout mice fed a protein deficient diet. The results suggest that adequate nutrition and ROS are essential for proper wound healing but excessive ROS cause delayed wound closure. This research will contribute to our understanding of the role of nutrition and ROS in wound healing and may lead to the development of better clinical strategies to enhance cutaneous wound healing.
2.2 INTRODUCTION

Wound healing is a physiological response to the disruption of normal tissue structure and function. The wound healing process can generally be divided into four stages - clot formation, inflammation, proliferation and remodeling. However, wound healing is an extremely complex process that involves the coordinated interaction of inflammatory cells, structural proteins, cytokines, growth factors and reactive oxygen species (ROS) (1-3).

Because wound healing is a cascade of processes, the inflammatory stage can be a regulatory step of later stages of wound healing (4). Indeed, the immunological phenomena of the inflammatory stage of wound healing have been well characterized. In particular, one of the initial events of the inflammatory stage is the infiltration of neutrophils and macrophages into the wound site. A primary function of both cell types is to clear contaminating cellular debris and bacteria through phagocytic processes that involve the production of ROS (5,6). In addition to its microbicidal activity, ROS also regulate to activate signal transduction pathway, to recruit inflammatory cells to the wound site, to stimulate release of proinflammatory agents such as cytokines and to modulate collagen synthesis during the wound healing process (7-10). Although ROS
serve many functions and are essential for the wound healing process, excessive ROS production can cause tissue damage and may in fact impair wound healing (7,11).

The deleterious effects of ROS are normally counteracted by the free radical or antioxidant defense system, a network of enzymatic and non-enzymatic components that quench or dismutate ROS and therefore reduce their destructive potential. For example, superoxide generated by inflammatory cells is converted to less reactive hydrogen peroxide by either copper-zinc superoxide dismutase (CuZnSOD) in the cytosol or manganese superoxide dismutase (MnSOD) in mitochondria. Indeed, it has been shown that SOD and glutathione peroxidase mRNA synthesis is significantly upregulated in the early stage of wound healing (5). However, enzymatic and non-enzymatic antioxidants have also been reduced during wound healing (10,11). Hence, the functional role of antioxidant defense in wound healing remains equivocal.

Whereas our understanding of the role of ROS in wound healing is still in its infancy, it is clear that nutritional status is a critical factor in this process. Indeed, sub-optimal nutritional status has been shown to prolong the stages of wound healing, and PEM in particular has been demonstrated to contribute to poor healing rates (12,13). Protein energy malnutrition (PEM) induces systemic as well as local immunodepression and also decreases protein synthesis during the wound healing process. Indeed, the decreased number and function in inflammatory cells (i.e. neutrophils, macrophages and lymphocytes) observed in PEM may prolong bacterial residence time and decrease production of inflammatory cytokines at the wound site (14-21). Therefore, nutrition plays a critical role in tissue repair and regeneration during wound healing.
Although it is known that nutritional status and ROS markedly affect the healing process, little research has been done to examine the effects of PEM and ROS on cutaneous wound healing. In these experiments we have hypothesized that PEM will increase the time of wound closure and decrease inflammatory cell infiltration into the wound site during the early inflammatory stage. In addition, because of the critical importance of ROS as mediators of cell signaling and phagocytic processes during wound healing, we have hypothesized that cutaneous wound healing will proceed more quickly in CuZnSOD knockout (CuZnSOD<sup>-/-</sup>), and will be delayed in CuZnSOD over-expresser (CuZnSOD<sup>+++</sup>) mice compared to CuZnSOD wild-type (CuZnSOD<sup>+/+</sup>) mice.
2.3 MATERIALS AND METHODS

Materials and Study Design

1) PEM study

CD-1 female mice (n=78), 4 weeks old, were obtained from Harlan Inc. (Indianapolis, IN) and used in accordance with animal protocols approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee. Animals were fed AIN-93G purified rodent powder diet (Dyets Inc., Bethlehem, PA) containing 0.5% (a protein deficient diet) or 15% protein (a control diet) as casein based isocaloric diet for two weeks. The ad-libitum control group was provided with free access to food. The pair fed group was fed the same amount of a control diet as the mice fed a protein deficient diet had in the previous day. Mice were randomized to two different sets for wound closure (n=18) and histology (n=60) respectively. Dietary intake was measured every day.

2) The Role of ROS Using CuZnSOD Transgenic Mice

To determine a potential effect of ROS using CuZnSOD transgenic mice (over-expressor, wild-type and knockout n=36, 6-8 weeks old) on wound healing wound closure was examined during cutaneous wound healing. CuZnSOD transgenic mice were
bred in our laboratory and used in accordance with animal protocols approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee. Animals were fed AIN-93G purified rodent powder diet (Dyets Inc., Bethlehem, PA) containing 0.5% (a protein deficient diet) or 15% protein (a control diet) as casein based isocaloric diet for two weeks. Body weights were measured twice a week in both studies.

**Wounding and Harvesting**

A full thickness excisional wound model was used to examine wound closure in these experiments. Neutrophil infiltration was examined in PEM study. After diet treatment, mice were anaesthetized with isoflurane. Back of each mouse was shaved using an electrical hair clipper and the area was sterilized using an alcohol swab. Two identical wounds were made by a 3.5mm sterile biopsy punch (Miltex, York, PA) on mouse back. Wounds were photographed every day and wound size was quantified using by Canvas program (Canvas 7 SE) in all experiments. Wound closure was expressed as a ratio of wound size divided by initial wound size. To investigate histological changes of tissues, mice in PEM study was euthanized with overdose of anesthesia and wounds, liver and lung were removed at 0, 12 hour (hr), day 1, day 2, day 3 and day 4 after wounding. Harvested tissues were immediately stored in 4% para-formaldehyde solution for histology.

**Histology**

Livers, lungs and wounds were fixed with 4% para-formaldehyde in phosphate-buttered saline (PBS, pH 7.4) for 0, 12 hr, day 1, day 2, day 3 and day 4 after wounding.
Tissues were then washed in PBS, dehydrated in a series of alcohols and embedded in paraffin. Hematoxylin and eosin (H&E) staining was performed to examine histological analysis of paraffin sections of tissues.

**Statistical Analysis**

Body weight and wound size were statistically evaluated by analysis of variance (ANOVA). Differences among groups were considered significant at p < 0.05.
2.4 RESULTS

1) PEM study

Body weight change and diet intake in PEM study

Food intake was 5.79g/day in PEM group and 6.85g/day in ad-libitum control group respectively and it was 14.5% calorie restriction in the pair fed control group (data not shown). Body weight loss was 18.1% in PEM group and was statistically significant compared to the pair fed group and the ad-libitum control group. Weight gains were 1.85% and 13.9% in pair fed control group and ad-libitum control group respectively (Figure 2.1).

Tissue morphology in PEM study

Morphological change was found in PEM hepatocytes (Figure 2.2). Decreased cytoplasmic contents and smaller nuclei of hepatocytes were examined in livers of PEM mice compared to those of the ad-libitum control group and the pair fed control group. There was no difference in lung morphology in PEM group (data not shown).

Wound closure in PEM study

Wound closure was statistically significant at day 1, day 6 and daily beginning at day 9 in PEM mice compared both the ad-libitum group as well as the pair-fed control
group. Although there was no statistical significance between days 2 through 5 significance (p-value) was less than 0.08 after performing ANOVA. PEM caused delayed wound closure. On the other hand, wound closure in the pair fed control group was statistically significant compared to that of a PEM group from day 7 to day 9 (p<0.05) (Table 2.1, Figure 2.3).

Neutrophil infiltration

The epithelial layer of skin tissue in PEM group was much thinner when compared to that of control mice. Neutrophil infiltration was delayed in PEM group compare to the ad-libitum control group and the pair fed control group at 12hr (Figure 2.4). Neutrophils stayed until day 4 in each group. However, macrophages were recruited with less neutrophils in the pair fed control group compared to the ad-libitum control group. Proliferation was not seen in PEM group and less proliferation was shown in the ad-libitum control group compared to the pair fed control group. Mature granulation and blood vessels were seen in wounded skin in the pair fed group at day 4.

2) The effect of ROS on wound closure in PEM

Body weight change

Body weight changes in CuZnSOD transgenic mice were statistically significant between the groups fed a protein deficient diet and the groups fed a control diet (Figure 2.5).
CuZnSOD knockout mice fed a control diet showed statistically faster wound closure than CuZnSOD over-expresser mice in the early inflammatory stage of cutaneous wound healing from day 1 to day 4 (p<0.05) (Table 2.2, Figure 2.6). However, delayed wound closure in CuZnSOD over-expresser mice was sped up since day 5. On the other hand, CuZnSOD knockout mice fed a protein deficient diet showed significantly delayed wound closure compared to CuZnSOD knockout mice fed a protein deficient diet since day 9 in the later stage of wound healing (p<0.05) (Table 2.3, Figure 2.7). Although there was no statistically significance CuZnSOD knockout mice fed a protein deficient diet had delayed wound closure from the early inflammatory stage of wound healing. Two different diets treatment showed the opposite result in the effect of ROS during cutaneous wound healing in the same genotype of CuZnSOD transgenic mice.
2.5 DISCUSSION

In the present study, we demonstrated that wound closure was delayed in PEM mice. Indeed, wound closure was significantly delayed during the early inflammatory stage in PEM mice compared to both the ad-libitum as well as the pair-fed control groups (Table 2.1, Figure 2.3). Moreover, wound closure was significantly slower in PEM mice compared to ad-libitum and pair-fed controls. Although wound size in ad-libitum control mice was consistently smaller than wound size in PEM mice during this same period, the differences were not statistically significant until day 11 and day 12. Regardless, these data indicate that wound healing, as measured by wound size, was clearly compromised in PEM mice. In addition, we also observed delayed neutrophil infiltration in PEM mice compared to both ad-libitum and pair-fed control mice at 12 hrs, but prolonged neutrophil residence at 96 hrs, further indicating that wound healing was impaired in PEM mice.

Wound healing is a dynamic process characterized by the interaction of numerous cell types and the various cytokines and extracellular proteins they produce. The results of this experiment clearly indicate that PEM disturbs the dynamics of wound healing to the point of significantly delaying wound closure. Although many factors may contribute to this phenomenon, we have demonstrated that delayed and decreased neutrophil infiltration may be a critical event that contributes significantly to delayed wound healing.
in PEM (Figure 2.4). Although it is well accepted that PEM impairs host immunity, particularly the T-cell system, this is the first histological documentation that PEM leads to decreased neutrophil infiltration during cutaneous wound healing. Because neutrophils play an essential role as both phagocytic cells and as cells that produce cytokines which signal subsequent steps in the wound healing process, decreased neutrophil infiltration may be a prominent factor that slows the wound healing process.

Another interesting finding of this study was that wound closure during the early inflammatory stage proceeded much more rapidly in CuZnSOD\(^{-/-}\) mice compared to CuZnSOD\(^{++/+}\) mice (Table 2.2, Figure 2.6). To our knowledge, this is the first demonstration that genetic deletion of the antioxidant enzyme CuZnSOD significantly increases cutaneous wound closure, when compared to mice that over-express this same enzyme, during the early inflammatory stage of wound healing. Although it is commonly understood that ROS serve mainly to kill bacteria and prevent infection at the wound site, recent evidence indicates that low-level ROS may serve as signaling molecules and induce the expression of genes involved in the wound healing process (22,23).

There is increasing evidence that wound repair can be facilitated by increasing the oxygen supply to the wounds (24). For example, clinical treatments utilizing hyperbaric oxygen therapy have shown that wound hyperoxia increases wound granulation tissue formation and accelerates wound closure (25,26). Nevertheless, the physiological bases for these observations remain largely unknown. Currently, reactive oxygen species (ROS) are being examined for their role in the wound healing process. ROS, primarily superoxide (O\(_2^−\)), are generated from oxygen during the respiratory burst and serve as microbicidal molecules that mediate cell death of foreign organisms. However, O\(_2^−\) is
also converted to hydrogen peroxide (H$_2$O$_2$), a compound that purportedly favorably influences signal transduction processes that support healing (27). Consistent with this hypothesis, our results demonstrate that wound healing occurs at a significantly greater rate in CuZnSOD$^{-/-}$ mice, where the ROS burden is presumably higher, than in CuZnSOD$^{+++}$ mice.

We also examined the effects of PEM on wound closure in CuZnSOD transgenic mice (Table 2.3, Figure 2.7). In particular, we found that wound closure during the early stage of wound healing was faster in CuZnSOD$^{-/-}$ mice fed a control diet, but was faster in CuZnSOD$^{+++}$ mice fed a protein deficient diet. Previously it has been demonstrated that CuZnSOD mRNA expression is dramatically increased at day 5 during the healing process, an observation consistent with the notion that ROS are critical during the early inflammatory phase, but may be deleterious during the later stages of wound healing (5,28). Based upon our results from mice fed a control diet in which wound closure was faster in CuZnSOD$^{-/-}$ than in CuZnSOD$^{+++}$ mice, we speculate that ROS are beneficial in cutaneous wound healing in healthy subjects. In contrast, results from our PEM study demonstrated a significantly delayed wound closure in CuZnSOD$^{-/-}$ compared to CuZnSOD$^{+++}$ mice. These results may indicate that the combination of PEM and CuZnSOD gene knockout exert an excessive ROS burden, resulting in delayed wound closure.
2.6 ACKNOWLEDGMENTS

We would like to thank Mark Levy and Yu-Huai Tsai for their assistance in the animal study and Wenmin Lai for histology work.
**Figure 2.1 Body weight changes of mice fed different diets**

Female CD-1 mice (n=78, 4 weeks of age) were randomized into 3 groups. Each group was fed a protein deficient (0.5% protein) or a control (15% protein) diet for 2 weeks respectively. The pair fed group was fed the same amount of food as a PEM group was in a previous day.

PEM (mice fed a protein deficient diet)
PF (mice fed pair fed control diet)
AL (mice fed a control diet ad libitum)
Figure 2.2 Liver morphology in PEM; H&E staining

A: PEM liver; B: Pair fed liver; C: control liver

Arrow a indicates decreased cytoplasmic content and arrow b indicates smaller nuclei
Table 2.1 Wound closure in mice a fed a protein deficient diet during cutaneous wound healing

Each group was fed a protein deficient (0.5% protein) or a control (15% protein) diet for 2 weeks. The pair fed group was fed the same amount of food as a PEM group was in a previous day. Wound size was statistically evaluated by one-way ANOVA (n=24). The values are expressed means ± S.E. with different superscript letters (a, b, and c) at p < 0.05. Ratio of wound size was calculated by wound size/ initial wound size. a, b, and c show statistically significant difference among groups.

PEM (mice fed a protein deficient diet)
PF (mice fed restricted amount of diet)
AL (mice fed a control diet ad libitum)

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Figure 2.3 Wound closure in mice a fed a protein deficient diet during cutaneous wound healing
Each group was fed a protein deficient (0.5% protein) or a control (15% protein) diet for 2 weeks. The pair fed group was fed the same amount of food as a PEM group was in a previous day. Wound size was statistically evaluated by one-way ANOVA (n=24). The values are expressed means ± S.E. with different superscript letters (a, b, and c) at p < 0.05. Ratio of wound size was calculated by wound size/ initial wound size. a, b, and c show statistically significant difference among groups.
PEM (mice fed a protein deficient diet)
PF (mice fed restricted amount of diet)
CONTROL (mice fed a control diet ad libitum)
Figure 2.4 Infiltration of inflammatory cells in wounds (H&E staining)
A: neutrophil infiltration at 12hr after wounding in control wound; B: neutrophil infiltration at 12hr after wounding in PEM wound; C: neutrophil infiltration at 12hr after wounding in pair fed wound; D: neutrophil stay and macrophage infiltration at day 4 after wounding in control wound E: neutrophil stay at day 4 in PEM wound; F: macrophage infiltration at day 4 after wounding in pair fed group
Arrow a: neutrophils in wound; b: macrophages in wound; c: granulation and reepithelialization
Figure 2.5 Body weight changes of mice fed a protein deficient diet or a control diet in Cu/ZnSOD transgenic mice Cu/ZnSOD transgenic mice (n= 24, 6-8 weeks of age) were randomized into 2 in each genotype group. Each group was fed a protein deficient (0.5% protein) or a control (15% protein) diet for 2 weeks respectively.

PEM (mice fed a protein deficient diet)
CON (mice fed a control diet)
CuZnSOD ^++ (CuZn superoxide over-expresser)
CuZnSOD ^+/+ (CuZn superoxide wild-type)
CuZnSOD ^-/- (CuZn superoxide knockout)
Cu/ZnSOD transgenic mice (n= 18, 6-8 weeks of age) were fed a control (15% protein) diet for 2 weeks. Wound size was statistically evaluated by one-way ANOVA. The values are expressed means ± S.E. with different superscript letters (a and b) at p < 0.05. Ratio of wound size was calculated by wound size/initial wound size. a, b, and c show statistically significant difference among groups.

CuZnSOD ^+++^ (CuZn superoxide over-expresser)
CuZnSOD ^+/+^ (CuZn superoxide wild-type)
CuZnSOD ^+/−^ (CuZn superoxide knockout)

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<th>Cu/ZnSOD ^+/−^</th>
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Table 2.2 Wound closure during cutaneous wound healing in Cu/Zn SOD transgenic mice fed a control diet
Figure 2.6 Wound closure during cutaneous wound healing in Cu/Zn SOD transgenic mice fed a control diet

Cu/ZnSOD transgenic mice (n=24, 6-8 weeks of age) were a control (15% protein) diet for 2 weeks. Wound size was statistically evaluated by one-way ANOVA. The values are expressed means ± S.E. with different superscript letters (a and b) at p < 0.05. Ratio of wound size was calculated by wound size/initial wound size. a, b, and c show statistically significant difference among groups.

CuZnSOD +++ (CuZn superoxide overexpressor)
CuZnSOD +/- (CuZn superoxide wild-type)
CuZnSOD +/- (CuZn superoxide knockout)
Cu/ZnSOD transgenic mice (n=24, 6-8 weeks of age) were fed a protein deficient diet for 2 weeks. Wound size was statistically evaluated by one-way ANOVA. The values are expressed as means ± S.E. with different superscript letters (a and b) at p < 0.05. Ratios of wound size were calculated by wound size/initial wound size. a, b, and c show statistically significant differences among groups.

CuZnSOD+++ (CuZn superoxide over-expressor)
CuZnSOD++ (CuZn superoxide wild-type)
CuZnSOD−− (CuZn superoxide knockout)

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Table 2.3 Wound closure during cutaneous wound healing in Cu/Zn SOD transgenic mice fed a protein deficient diet
Figure 2.7 Wound closure during cutaneous wound healing in Cu/Zn SOD transgenic mice fed a protein deficient diet

Cu/ZnSOD transgenic mice (n=18, 6-8 weeks of age) were a protein deficient (0.5%) diet for 2 weeks. Wound size was statistically evaluated by one-way ANOVA. The values are expressed means ± S.E. with different superscript letters (a and b) at p < 0.05. Ratio of wound size was calculated by wound size/initial wound size. a, b, and c show statistically significant difference among groups.

CuZnSOD +++ (CuZn superoxide over-expresser)
CuZnSOD +/- (CuZn superoxide wild-type)
CuZnSOD +/- (CuZn superoxide knockout)
2.7 REFERENCES


CHAPTER 3

N-ACETYL CYSTEINE SUPPLEMENTATION NORMALIZES EARLY INFLAMMATORY STAGE OF CUTANEOUS WOUND HEALING IN PROTEIN ENERGY MALNOURISHED MICE

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¹Department of Human Nutrition, ²Department of Oral Biology, The Ohio State University, Columbus, OH and ³Linus Pauling Institute, Oregon State University, Corvallis, OR, USA
3.1 SUMMARY

Protein energy malnutrition (PEM) is a well-known health problem in developing countries. In affluent countries, PEM secondary to chronic disease is a prevalent crisis in hospitals. Furthermore, delayed wound healing in post-operative PEM patients is a known predicament.

The normal wound healing process consists of four major stages (clot formation, inflammation, proliferation and remodeling) involving the interactions and coordination of various inflammatory cells, extracellular matrix molecules, cytokines, chemokines and proteinases. Neutrophils and macrophages recruited during the early inflammatory stage play critical roles in clearing contaminating bacteria by producing reactive oxygen species (ROS). These inflammatory cells also produce proinflammatory cytokines that can stimulate immune gene transcription through NFκB activation.

PEM impairs antioxidant defense by reducing antioxidant enzymes and nutrients as well as compromised immune function. Therefore, a balanced nutritional status that modulates antioxidant defense and immune function becomes a determining factor for normal wound healing. N-acetyl cysteine (NAC) has been known to increase antioxidant defense and immune function by the increasing the concentration of glutathione.
We hypothesized that NAC supplementation would normalize the early inflammatory response and enhance cutaneous wound healing in PEM mice. We used a full-thickness excisional wound model to examine wound closure, neutrophil infiltration and gene expression of IκB, IL-1β and TNF-α during cutaneous wound healing. PEM mice showed delayed wound closure, impaired inflammatory cell infiltration and decreased gene expression of IκB, IL-1β and TNF-α compared to the control mice. NAC supplementation enhanced wound closure, neutrophil infiltration and increased gene expression of IκB, IL-1β and TNF-α during the early inflammatory stage of cutaneous wound healing in PEM mice. These data suggest that NAC supplementation during early stage of wound healing in PEM mice before nutritional intervention may have beneficial effects.
3.2 INTRODUCTION

Protein energy malnutrition (PEM) is a well-known nutritional health problem associated with high mortality and morbidity in developing countries. In affluent countries, PEM secondary to chronic disease is a prevalent crisis in hospitals (1). The high morbidity and mortality associated with PEM may be attributable to impaired immune function and antioxidant defense. Although the exact mechanisms are not clear, PEM may impaire immune function by reducing hematopoiesis and lymphopoiesis as well as by decreasing leukocyte activation and phagocytic activity (2,3). In addition, PEM may also impair antioxidant defense by reducing antioxidant vitamin status as well as the activity of proteins involved in antioxidant defense such as glutathione peroxidase and ceruloplasmin (4).

In addition to impaired immune function and antioxidant defense, delayed wound healing is a prominent characteristic of PEM and may be a significant contributor to the poor prognosis of PEM patients (5). Wound healing is a complex process that involves the interaction and coordination of many cell types, cytokines, extracellular matrix molecules, proteinases and even reactive oxygen species (ROS) (6,7). Despite its complexity, wound healing can generally be divided into four major stages: clot formation, inflammation, proliferation and remodeling. In particular, inflammation is
regarded as a critical stage that may control later stages of wound healing. Indeed, modulation of the inflammatory stage may be key to enhancing the wound healing process in PEM patients (8).

During the early inflammatory stage, neutrophils and macrophages are recruited to the wound site by chemotactic factors. At this stage, neutrophils and macrophages play critical roles in ROS mediated phagocytosis and in the production of pro-inflammatory cytokines (9). For example, interleukine (IL)-1\(\beta\) and tumor necrosis factor (TNF)-\(\alpha\) released by these cells recruit more inflammatory cells, enhance phagocytosis and stimulate the activation and proliferation of fibroblasts (10-13). In addition, pro-inflammatory cytokines stimulate the activation of nuclear factor kappa B (NF\(\kappa\)B), a redox-sensitive transcription factor that regulates gene expression of immune and inflammatory factors including cytokines, chemotactic factors and cell adhesion molecules (14-16). Since proinflammatory cytokines regulate NF\(\kappa\)B activation and are regulated by NF\(\kappa\)B through feed-back and feed-forward amplification, dysregulated production of proinflammatory cytokines may impair the wound healing process (17,18). Thus, the central role of NF\(\kappa\)B in the regulation of cytokines and cell adhesion molecules may point to its critical role as a modulator of the inflammatory stage of wound healing.

ROS as well as proinflammatory cytokines are a well known stimulator to activate NF\(\kappa\)B (17). ROS are beneficial to the initial wound healing process, however, excessive ROS can cause tissue damage and impair wound healing (19-21). Therefore, oxidants and antioxidants must be balanced in order to maintain a tight regulation of enzymatic and non-enzymatic antioxidant systems. There has been demonstrated the role of antioxidant defense system in injury. Non-enzymatic or enzymatic antioxidants are
declined during healing process but mRNA expression of antioxidant enzymes are upregulated after wounding (22-24). Therefore, nutritional status which modulates immune function and antioxidant defense can become determining factors for normal wound healing.

In PEM patients, nutritional intervention is needed to enhance immune function and antioxidant defense in the short-term period. However, a high protein or high calorie diet is not recommended to PEM patients during initial stage of rehabilitation as it may be toxic due to changes of body metabolism in PEM subjects (25). Therefore, therapeutic strategies for treatment of PEM patients at the early stage of rehabilitation have been focused on enhancing immune function and antioxidant defense in the absence of a high protein or energy rich diet. In particular, glutathione (GSH, L-γ−L-glutamyl-L-cysteinyl-glycine) has been investigated in PEM subjects in many studies. GSH is involved in drug detoxification, maintenance of intracellular protein integrity, regulation of signal transduction pathways and immune defense (26). Previous studies have demonstrated that GSH level is decreased in tissues such as liver and lung of PEM patients and animals and it is associated with impaired immune function and increased susceptibility to infection (4,27-29). Hence, elevating tissue GSH levels may enhance immune function and rehabilitation of PEM patients.

In our previous study, we observed increased wound closure time, delayed and prolonged neutrophil infiltration, as well as decreased macrophage infiltration during the inflammatory stage of cutaneous wound healing in PEM mice (unpublished data). Therefore in this study we examined the effect of nutritional supplementation of the cysteine pro-drug, NAC on indices of wound healing in PEM mice. Although cysteine is
the limiting amino acid of GSH synthesis, we have selected NAC because it has proven effective in elevating tissue GSH levels and exhibits much less toxicity than cysteine (30,31). In particular, we hypothesized that dietary supplementation of NAC would increase the expression of the proinflammatory cytokines IL-1β and TNF-α, increase the expression of inhibitory kappa B (IκB), a down stream gene of NF-κB, and increase the rate of wound closure during the early stage of cutaneous wound healing in PEM mice.
Materials

CD-1 female mice (n=81), 4 weeks old, were obtained from Harlan Inc. (Indianapolis, IN) and used in accordance with animal protocols approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee. Animals were fed AIN-93G purified rodent powder diet (Dyets Inc., Bethlehem, PA) containing 0.5% or 15% protein as a casein based isocaloric diet. NAC was obtained from Sigma Chemical Company (St.Louis, MO).

Mice were randomized to two different subgroups for wound closure (n=21) and histology (n=60) respectively. Immunofluorescence kits were obtained from Dako Company (Carpinteria, CA). Probes for mRNAs of IL-1β, TNF-α and IκB were provided by Dr. Quan.

Study Design

To investigate the effect of dietary supplementation of NAC on the early stage of cutaneous wound healing in PEM mice, mice were fed a 0.5% protein (a protein deficient diet) or a 15% protein diet (a control diet) respectively for 2 weeks and mice fed a protein deficient diet were randomized into 2 groups.
One group of mice fed a protein deficient diet and the mice fed a control diet were continued their diets. The other group of mice fed a protein deficient diet was fed a protein deficient diet supplemented NAC. The amount of NAC added in a protein deficient diet was 59.5 mmol/kg diet which was the same amount of sulfur amino acids in a control diet for another week. Food intake was determined every other day and body weights were measured twice a week.

**Wounding and Harvesting**

A full thickness excisional wound model was used to examine wound closure, neutrophil infiltration and mRNA expression of IκB, TNF-α and IL-1β. After a week supplementation of NAC, mice were anaesthetized with isoflurane. Back hair of each mouse was shaved using an electric hair clipper and the area was sterilized using an alcohol swab. Two identical wounds were made by a 3.5mm sterile biopsy punch (Miltex, York, PA) on the back. One set of mice for wound closure, was photographed every day and wound size was quantified using Canvas program (Canvas 7 SE). Wound closure was expressed as a ratio of wound size compared to the initial wound size. Wound appearance was examined based on the pictures taken. The other set of mice was euthanized with overdose of anesthesia and wounds, liver and lung were removed for histology at 0 hour (hr), 6 hr, 12 hr, day 1 and day 2 after wounding. Harvested tissues were immediately stored in 4% para-formaldehyde solution for histology and *in situ* hybridization.
**Histology**

Livers and wounds were fixed with 4% para-formaldehyde in phosphate-buttered saline (PBS, pH 7.4) for 0 hr, 6 hr, 12 hr, day 1 and day 2 after wounding. Tissues were then washed in PBS, dehydrated in a series of alcohols and embedded in paraffin. Hematoxylin and eosin staining was performed to examine histological analysis of paraffin sections of tissues.

**In Situ hybridization (Immunofluorescence)**

The *in situ* hybridization protocols were performed as described previously (32). Paraffin embedded tissue sections were dewaxed in xylene, and dehydrated in a series of different concentrations of ethanol. Dewaxed slides were incubated in pepsin (150µl/ml), fixed with 4% formaldehyde solution, and acetylated with 0.25% acetic anhydride in 0.1M triethanolamine-HCl. Antisense probes were transcribed using the Riboprobe Systm (Promega Biotech, Madison, WI) with T7 RN polymerase. IκBα cDNA, TNF-α cDNA and IL-1β cDNA were generously provided by Dr. Quan. To control probe specificity, sense probes were generated by transcribing the same plasmid with T3 RNA polymerase. Lastly, probes were diluted in the riboprobe hybridization buffer and applied to wound tissue section slides. After overnight incubation at 55°C in a humidified chamber, slides were washed first in 2X, 1X, and 0.2X SSC at 55°C. They were then incubated in a blocking buffer for 30 min before the diluted anti-DIG antibody was added onto the slides and incubated for 1 hr. Then, they were incubated with anti-mouse antibody for 1 hr, primary Streptavidin for 15 min, BT solution for 15 min and
CY2-Streptavidin for 1 hr in dark by order. The slides were viewed with microscope through fluorescence filter.

**Statistical Analysis**

Body weight and wound size were statistically evaluated by analysis of variance (ANOVA) and significant differences among groups were considered at $p < 0.05$. 
3.4 RESULTS

Diet intake and Body weight

Dietary intake was not statistically different among the treatment groups (data not shown). However, although mice fed the control diet exhibited weight gain throughout the study, mice fed the PEM diet lost weight throughout the 3 week treatment. Body weight of PEM mice was statistically different from that of control mice beginning on day 3 (Figure 3.1).

Tissue Morphology

PEM caused morphological changes in liver hepatocytes. Decreased cytoplasmic contents and smaller nuclei were observed in PEM mice compared to those of control mice. NAC supplementation prevented shrinking nuclei but did not restore cytoplasmic content (Figure 3.2).

Wound Closure

Color of wounds in PEM mice was yellowish and scars of PEM were obviously larger and deeper compared to those of PEM mice with NAC supplementation and of control mice during early stage of wound healing (Figure 3.3). However, the appearance
of wounds in PEM mice and PEM mice with NAC supplementation was not normalized
during later stages of wound healing.

Wound closure in PEM mice was statistically different from those of PEM mice
with NAC supplementation and control group at day 1 and day 2 after wounding during
cutaneous wound healing (Table 3.1, Figure 3.4). In addition, PEM mice with NAC
supplementation showed a similar pattern of wound closure as that of control mice by day
4 after wounding (Figure 3.5). However, beginning on day 5, wound closure of PEM
mice was statistically different from that of control mice. Similarly, beginning on day 5,
wound size of PEM mice with NAC supplementation as well as PEM mice was
statistically greater than that of mice fed a control diet.

Neutrophil Infiltration

The effects of NAC supplementation on neutrophil infiltration in cutaneous
wounds in mice were investigated. Neutrophil infiltration was delayed in PEM mice
compared to a control mice but NAC supplementation in PEM mice normalized
neutrophil infiltration at 12 hr and 24 hr after wounding (Figure 3.6).

The Effects of NAC on the Expression of I\(\kappa\)B

To investigate the activation of NF\(\kappa\)B indirectly in wounds, the expression of I\(\kappa\)B
was detected by in situ hybridization. Low-level I\(\kappa\)B mRNA in PEM mice was detected
compared to that of control mice at day 1 and day 2 after wounding. However, NAC
supplementation restored the expression of I\(\kappa\)B mRNA in PEM mice to levels similar to
that observed in control mice (Figure 3.7).
The Effects of NAC on the Expression of IL-1β and TNF-α

Most mRNA expression of IL-1β was detected in the dermis at the wound edge and in the clot. Lower expression of IL-1β mRNA in PEM mice was detected compared to that of control mice at 12 hr and day 1 after wounding. Furthermore, NAC supplementation increased mRNA expression of IL-1β in PEM mice (Figure 3.8). Low-level expression of TNF-α mRNA was observed in all treatment groups in each time point. However, TNF-α mRNA in PEM mice was hardly expressed at all time points but NAC supplementation normalized expression of TNF-α mRNA in PEM mice at 12 hr after wounding (Figure 3.9).
3.5 DISCUSSION

Wound healing is a complex process that involves the coordinated interaction and of many different cell populations and the numerous factors they produce. In PEM patients, impairment of the wound healing process may contribute to their high rate of morbidity and mortality. In these experiments, we demonstrated that dietary supplementation of NAC in PEM mice enhanced the rate of wound closure, increased neutrophil infiltration, and increased mRNA expression of IκB, IL-1β and TNF–α during the early inflammatory stage of wound healing.

Assessment of changes in wound size is the most commonly reported measure of wound healing in the clinical setting. Indeed, wound size is widely regarded as an indicator of the severity as well as the prognosis of the wound, and it is considered to be both an objective and widely applicable measure of the healing process (33). Although complete wound healing would be the ideal endpoint of wound healing assessment, this is not always feasible and therefore partial wound closure provides a reasonable alternative. We have demonstrated that cutaneous wound size was greater in PEM mice compared to control mice during the early inflammatory stage (Table 3.1, Figure 3.4 and Figure 3.5). Moreover, we have shown that NAC supplementation during the early inflammatory stage provided significant benefit, reducing the wound size in PEM mice to control
values. Thus, NAC supplementation may provide significant benefit in terms of enhancing wound closure during the early rehabilitation of PEM patients and may therefore considerably improve the prognosis of PEM patients in the clinical setting. However, NAC supplementation didn’t show benefit since day 5 it may be due to lack of protein and amino acids for proliferation and remodeling. Hence, adequate protein intake after the early inflammatory stage should be recommended for proper wound healing.

Recruitment of inflammatory cells for phagocytic activity and cytokine production to the wound site immediately after wounding is considered an essential process for proper wound healing. Indeed, the early inflammatory stage may be the most critical stage of wound healing that ultimately dictates the progression of subsequent stages. During the early stages of inflammation, neutrophils provide the first line of nonspecific defense and are rapidly recruited within a few minutes after wounding to clear contaminating bacteria and eliminate foreign material through phagocytosis (34). Although neutrophil numbers soon decline, the influx of neutrophils during early inflammation is critical to prevent the development of infection and promote timely wound healing (35). Results from this work demonstrated that neutrophil infiltration into the wound site was delayed in PEM mice (Figure 3.6). However, we also observed that NAC supplementation to PEM mice normalized neutrophil infiltration to levels equal to that observed in control mice. In our previous study, neutrophil infiltration to the wound site was delayed, and neutrophil residence time was considerably longer in PEM mice compared to controls (unpublished data). These results suggested that protein energy deficiency prolong inflammation and may adversely affect the normal healing process. The delayed infiltration and longer residence time of neutrophils may be attributable to
their impaired chemotactic factor synthesis that reduces macrophage recruitment, or it may be due to defects of macrophage receptors that are essential for their recruitment to the wound site. Regardless, failure of macrophage recruitment may significantly prolong neutrophil infiltration, as macrophages function not only to induce neutrophil apoptosis, but also to ingest the apoptotic neutrophils. More critically, depressed macrophage recruitment has been shown to significantly impair the healing process (36).

NFκB participates in the up-regulation of a diverse range of genes involved in inflammation and acute phase response. NFκB is constitutively present in the cytoplasm in an inactive form, bound to the inhibitory protein kappa Bα (IkBα). Upon activation, IkBα is degraded, and NFκB translocates to the nucleus and regulates the expression of inflammatory genes i.e. cytokines, chemotactic factors and cell adhesion molecules (14-16,37). NFκB also upregulates IκBα mRNA expression and, through a negative regulatory feedback loop, newly synthesized IκBα protein then serves to inhibit NFκB activity. Hence, IκBα expression can serve as an indirect indicator of NFκB activation.

In our experiments, we observed decreased IκBα expression in wound tissue of PEM mice compared to control mice (Figure 3.7). However, NAC supplementation of PEM mice elevated IκBα expression to levels intermediate to that observed in PEM and control mice. We propose that the reduced expression of IκBα mRNA, and therefore presumably decreased activation of NFκB, observed in PEM mice may adversely affect proinflammatory cytokine expression and hence impair the wound healing process. We further propose that the orderly and timely activation of NFκB is critical to promote normal cutaneous wound healing in PEM.
Proinflammatory cytokine production (e.g. IL-1 and TNF-α) has been shown to be decreased in PEM children and may be a significant factor that contributes to the delayed wound healing observed in this population (38,39). For example, the cytokine TNF-α is a primary proinflammatory cytokine that plays an integral role in neutrophil mediated superoxide production and phagocytosis. It may also be an important cytokine that induces neutrophil apoptosis (40,41). Similarly, IL-1β functions as an activator of neutrophils and a chemotactic factor that induces fibroblast granulation and collagen synthesis and is therefore critical to the development of wound tensile strength. Hence, dysregulation of cytokine production may have devastating effects on the wound healing process (10,42). In our experiments, we have demonstrated that mRNA production of IL-1β and TNF–α was delayed in PEM mice compared to control mice at 12 hr and 24 hr after wounding (Figure 3.8). However, NAC supplementation restored gene expression of IL-1β and TNF–α in PEM mice. Based upon these results, we speculate that the delayed wound healing observed in PEM mice may in part be attributable to the decreased expression of IL-1β and TNF–α. Because of their biological roles as an activator of neutrophil or an stimulator of fibroblast and keratinocyte growth, and because IL-1β functions in both the synthesis and breakdown of extracellular matrix proteins, it is reasonable to speculate that decreased expression of these cytokines may delay the wound healing process by impairing tissue regeneration and therefore delay restoration of normal tissue structure and function.

In summary, we observed increased wound closure time, decreased neutrophil infiltration, as well as decreased expression of IκBα, IL-1β and TNF–α during the early
inflammatory stage in wound tissue of PEM mice compared to control mice.

Furthermore, we demonstrated that NAC supplementation of PEM mice decreased wound closure time, increased neutrophil infiltration and also increased IkBα, IL-1β and TNF-α expression compared to PEM mice. These findings provide evidence for the use of NAC supplementation as therapeutic strategy to enhance wound healing during the early inflammatory stage in PEM patients. In particular, these results may provide critical insight into future nutritional intervention strategies designed to enhance immune function not only in patients suffering from PEM, but also in patients suffering from malnutrition related disorders.
3.6 ACKNOWLEDGMENTS

We would like to thank Mark Levy and Yu-Hwai Tsai for their assistance in the animal study and Wenmin Lai for histology work.
Figure 3.1 Body weight changes of mice fed different diets
Female CD-1 mice (n= 81) were randomized into 3 groups. Each group was fed a protein deficient (0.5% protein) or a control (15% protein) diet for 2 weeks. The group fed a protein deficient diet was randomized to two groups and one group was fed a protein deficient diet the other group of mice was fed a protein deficient diet supplemented N-acetyl cysteine (NAC). The mice fed a control diet were continued their diets.
PEM (mice fed a protein deficient diet)
PEM + NAC (mice fed a protein deficient diet with NAC supplementation)
CON (mice fed a control diet)
Figure 3.2 Liver morphology in PEM with NAC supplementation; H&E staining
A: PEM liver; B: PEM liver with NAC supplementation; C: control liver
Arrow a indicates decreased cytoplasmic content (fat degeneration); arrow b indicates smaller nucleus
Figure 3.3 Cutaneous wounds in PEM with NAC supplementation
A: PEM wound; B: PEM wound with NAC supplementation; C: control wound
### Table 3.1 The effects of NAC supplementation on wound closure during cutaneous wound healing in PEM mice

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<th>PEM</th>
<th>PEM+NAC</th>
<th>CON</th>
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<tr>
<td>Day 0</td>
<td>1</td>
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<td>1</td>
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<td>.90±.06&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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<td>.88±.07</td>
<td>.84±.06</td>
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<tr>
<td>Day 4</td>
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<td>.84±.07</td>
<td>.83±.06</td>
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<td>.82±.07&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>.60±.07&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>.74±.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.38±.05&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>.70±.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.23±.04&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Day 8</td>
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<td>.56±.07&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>.33±.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
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Each group was fed a protein deficient (0.5%) or a control (15%) diet for 2 weeks. The group fed a protein deficient diet was randomized to two groups. One group was fed a protein deficient diet and the other group fed a protein deficient diet with N-acetyl cysteine (NAC) supplementation for another one week before wounding. The mice fed a control diet were continued their diets. Wound ratio was statistically evaluated by one-way ANOVA (n=21). The values are expressed means ± S.E. with different superscript letters (a and b) at p < 0.05.

Ratio of wound size was calculated by wound size/ initial wound size. a and b show statistically significant difference among groups.

PEM (mice fed a protein deficient diet)
PEM+NAC (mice fed a protein deficient diet with NAC supplementation)
CON (mice fed a control diet)
Figure 3.4 The effects of NAC supplementation on wound closure during cutaneous wound healing in PEM mice
Wound ratio was statistically evaluated by one-way ANOVA (n=21). The values are expressed means ± S.E. with different superscript letters (a and b) at p < 0.05.
Ratio of wound size was calculated by wound size/ initial wound size. a and b show statistically significant difference among groups.
PEM (mice fed a protein deficient diet)
PEM+NAC (mice fed a protein deficient diet with NAC supplementation)
CON (mice fed a control diet)
Figure 3.5 The effects of NAC supplementation on wound closure during the early stage of cutaneous wound healing in PEM mice

Wound ratio was statistically evaluated by one-way ANOVA (n=21). The values are expressed means ± S.E. with different superscript letters (a and b) at p < 0.05.

Ratio of wound size was calculated by wound size/initial wound size. a and b show statistically significant difference among groups.

PEM (mice fed a protein deficient diet)
PEM+NAC (mice fed a protein deficient diet with NAC supplementation)
CON (mice fed a control diet)
Figure 3.6 Neutrophil infiltration in PEM with NAC supplementation (H&E staining)
Arrows indicate neutrophil infiltration in wounds
Figure 3.7 IκBα expression in PEM with NAC supplementation (In Situ Hybridization)
Fluorescence density indicates the amount of IκBα expression in wounds
Figure 3.8 IL-1β expression in PEM with NAC supplementation (*In Situ* Hybridization)
Fluorescence density indicates the amount of IL-1β expression in wounds
Figure 3.9 TNF-α expression in PEM with NAC supplementation (In Situ Hybridization) at 12hr after wounding
Fluorescence density indicates the amount of TNF-α expression in wounds
3.7 REFERENCE


CHAPTER 4

THE EFFECT OF DIETARY ZINC ON THE EARLY INFLAMMATORY STAGE DURING CUTANEOUS WOUND HEALING

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4.1 SUMMARY

Wound healing is a multifactorial process that consists of four major stages – clot formation, inflammation, proliferation and remodeling. Nutritional status affects healing process by changing of immune response and antioxidant defense. The healing process requires nutrients to provide energy and substrates for proper repair and regeneration. During wound healing, inflammatory stage may be the most important because neutrophils and macrophages are recruited and play critical roles in the production of pro-inflammatory cytokines. Proinflammatory cytokines attract other cells and activate the gene transcription factor, NFκB, which in turn regulates the expression of numerous genes during the inflammatory stage. Therefore, the regulation of NFκB may be critical for wound healing.

Zinc is a well-known antioxidant and micronutrient related to immune function. Inadequate zinc intake is characterized by delayed wound healing and impaired immune function. In these experiments we have hypothesized that zinc deficiency would delay the early inflammatory stage of wound healing. Specifically, we hypothesized that zinc deficiency would delay wound closure by decreased neutrophil infiltration and decrease activation of NFκB, a redox regulated transcription factor but zinc supplementation would help to accelerate wound closure by enhanced neutrophil infiltration and increased
activation of NFκB during cutaneous wound healing. We used a full-thickness excisional wound model in mice to examine neutrophil infiltration, gene expression of IκB (indirect measurement of NFκB), IL-1β and TNF-α in wounds and the time of wound closure in mice fed a diet containing 0 (deficient), 50 (control), 500 or 1000 ppm zinc diet. Zinc deficiency caused delayed neutrophil infiltration, decreased expression of IκB (or NFκB) and significantly delayed wound closure compared to control mice. On the other hand, zinc supplementation at 500 ppm zinc accelerated neutrophil infiltration and increased expression of IκB and wound closure compared to other groups. However, zinc supplementation at 1000 ppm delayed neutrophil infiltration, led to decreased expression of IκB and significantly delayed wound closure. These data suggest that zinc deficiency and high dose zinc supplementation delayed wound healing through decreased NFκB activation. Moreover, adequate zinc supplementation may have beneficial effects in the early inflammatory stage to accelerate cutaneous wound healing though increased NFκB activation.
4.2 INTRODUCTION

Wound healing is a complicated network that involves the interaction and coordination of various cell types, structural proteins, cytokines, chemokines, and reactive oxygen species (ROS) (1-4). Although is a highly dynamic process, it can be generally divided into four major stages – clot formation, inflammation, proliferation and remodeling. Following clot formation, the early inflammatory stage is initiated with the infiltration of neutrophils and macrophages, the major inflammatory cells that serve to clear contaminating bacteria and cellular debris through phagocytosis and production of ROS (5). These inflammatory cells secrete proinflammatory cytokines and growth factors as well as ROS that signal to recruit and activate the other cells such as fibroblasts (6). Proinflammatory cytokines play crucial roles in activating immune genes such as cytokines and growth factors that are regulated by a redox-sensitive gene transcription factor, nuclear factor kappa B (NFκB) (7,8). Therefore, because wound healing is a cascade of processes, the events of the early inflammatory stage may be critically important to progression of subsequent wound healing processes.

The wound healing process can be greatly influenced by nutritional status. This is exemplified by two observations. First, malnutrition increases the risk for wound-related complications and significantly increases wound-healing time. Second, dietary
intervention, either through complete nutritional support or single nutrient supplementation, can dramatically improve and accelerate the wound healing process (9). Notably, nutritional status can have a profound effect on numerous aspects of wound healing, including immune function and antioxidant defense (10). Indeed, a balanced nutrition is deemed critical for normal repair and regeneration during wound healing (4) (reviewed by (11,12).

Zinc deficiency is a well-known health problem associated with chronic diseases such as cancer, renal diseases, alcoholism and gastrointestinal disorders in United States. Zinc deficiency is characterized by growth retardation, susceptibility to infection and, importantly, poor wound healing (13). In particular, zinc deficiency is related to impaired innate and acquired immune function, including impaired leukocyte and lymphocyte function as well as altered cytokine production (13-16). The effect of zinc in the wound healing process has been investigated since the 1950’s (17). Zinc deficiency has been shown to increase wound closure time and decrease wound strength (18,19). Notably, zinc has been used as a topical agent to treat diaper rash, and it has also been used as a nutritional supplement in patients with bedsores, ulcers and incisional wounds. Several studies have also shown that zinc concentration is decreased in serum and at the wound edge during the proliferative phase of wound healing (20,21). In fact, topical treatment of zinc oxide and zinc tape are beneficial in wound closure and collagen synthesis in wound healing (22-25). These findings indicate not only that zinc deficiency can deleteriously affect the wound healing process, but also that zinc supplementation, either through topical application or dietary intake, can enhance the wound healing process.
One potential mechanism through which zinc may influence wound healing is through its effects on NFκB, a redox-sensitive transcription factor that regulates a vast array of inflammatory genes. Recently, zinc deficiency has been shown to reduce NFκB nuclear binding activity \textit{in vitro} and \textit{in vivo} \cite{26,27}. Moreover, zinc deficiency has been shown to decrease production of IL-2, a cytokine containing an NFκB binding sequence in its promoter region \cite{28}. Hence, there is limited evidence that zinc deficiency may impair wound healing through its adverse effects on NFκB DNA binding activity.

In these experiments, we have investigated the effects of zinc deficiency on the early inflammatory stage of wound healing. In particular, we have investigated the effects of zinc deficiency on NFκB activation, proinflammatory cytokine production, neutrophil infiltration and wound closure during the early inflammatory stage of wound healing. We have hypothesized that zinc deficiency will adversely affect NFκB binding activity, leading to impairments in proinflammatory cytokine production, neutrophil infiltration and wound closure in a murine model of cutaneous wound healing. Moreover, we propose that dietary zinc supplementation with super-physiological levels of zinc may enhance and accelerate the wound healing process in this model.
4. 3 MATERIALS AND METHODS

Materials

CD-1 female mice (n=120), 3 weeks old, were obtained from Harlan Inc. (Indianapolis, IN) and used in accordance with animal protocols approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee. Animals were fed AIN-76G purified rodent powder diet (Dyets Inc., Bethlehem, Pennsylvania) containing 0 ppm, 50 ppm, 500 ppm or 1000 ppm zinc as an egg white based protein diet. Mice were randomized mice into two different sets for wound closure and histology respectively. Immunofluorescence kits for in situ hybridization were obtained from Dako Company (Carpinteria, CA). Probes for mRNA of IL-1β, TNF-α and IκB were provided by Dr. Ning Quan (OSU).

Study Design

To examine the effect of dietary zinc on the early inflammatory stage of cutaneous wound healing, mice were fed AIN-76G purified rodent diet (Dyets Inc, Bethlehem, PA) containing 0 (zinc deficient diet), 50 (control diet), 500 or 1000 ppm zinc respectively for 2 weeks. Mice were randomized two different sets for wound
closure and histology respectively. Zinc free metal wire cages, water bottles and food jars washed with EDTA were used to prevent zinc contamination from water, cages and water bottles. Food intakes and body weights were measured every other day.

Wounding and Harvesting

A full thickness excisional wound model was used to examine wound closure, neutrophil infiltration and mRNA expression of IκB, TNF-α and IL-1β after two-week diet treatment. Mice were anaesthetized with isoflurane after diet treatment. The mouse back was shaved using an electric hair clipper and the area was sterilized using an alcohol swab. Two identical wounds were made by a 3.5mm sterile biopsy punch (Miltex, York, PA) on the back. One set of mice for wound closure, was photographed every day and wound size was quantified using Canvas program (Canvas 7 SE). Wound closure was expressed as ratio calculated by initial wound and internal standard circle. Wound appearance was examined based on the pictures taken. The other set of mice was euthanized with overdose of anesthesia and then blood was taken and wounds were removed for histology at 0, 6, 12, 24 and 48 hr after wounding. Harvested wounds were immediately stored in liquid N₂ (only 0 hr and 24 hr) for skin zinc concentration and in 4% para-formaldehyde solution for histology and in situ hybridization.

Zinc Concentrations in Serum and Skin

Serum was separated by centrifuge at 2000g for 10 min. Zinc concentration in serum or skin were assessed according to the method by Literati et al (29). Serum or skin tissues were digested in 2 N hydrochloric acid for 24 hr at room temperature. Samples
were then centrifuged at 7000g for 25 min, and the supernatant was used for direct measurement of metal concentrations using an atomic absorption spectrometer (Model AA-5, Varian Techtron, Australia).

**Histology**

Wounds were fixed with 4% para-formaldehyde in phosphate-buttered saline (PBS, pH 7.4) for 0, 6, 12, 24 and 48 hr after wounding. Wound tissues were then washed in PBS, dehydrated in a series of alcohols and embedded in paraffin. Hematoxylin and eosin (H&E) staining was performed to examine histological analysis of paraffin sections of tissues.

**In Situ hybridization (Immunofluorescence)**

The *in situ* hybridization protocols were performed as described previously (30). Briefly, paraffin embedded tissue sections were dewaxed in xylene and dehydrated in a series of different concentrations of ethanol. Dewaxed slides were incubated in pepsin (150µl/ml), fixed with 4% para-formaldehyde solution and acetylated with 0.25% acetic anhydride in 0.1M triethanolamine-HCl. Antisense probes were transcribed using the Riboprobe System (Promega Biotech, Madison, WI) with T7 RN polymerase. IκB cDNA, TNF-α cDNA and IL-1β cDNA were generously provided by Dr. Quan. To control for specificity of the probe, sense probes were generated by transcribing the same plasmid with T3 RNA polymerase. Lastly, probes were diluted in the riboprobe hybridization buffer and applied to wound tissue section slides. After overnight
incubation at 55°C in a humidified chamber, slides were washed first in 2X, 1X, and 0.2X SSC at 55°C. They were then incubated in a blocking buffer for 30 min before diluted anti-DIG antibody was added onto the slides and incubated for 1 hr. Then they were incubated with anti-mouse antibody for 1 hr, primary Streptavidin for 15 min, BT solution for 15 min and CY2-Streptavidin for 1 hr in dark by order. The slides were viewed with microscope through fluorescence filter.

**Statistical Analysis**

Body weight and wound size were statistically evaluated by analysis of variance (ANOVA). Significant differences among groups were considered at p < 0.05.
4.4 RESULTS

Body Weight and Food Intake

Weight change and diet intake among all groups for two-week treatment were not statistically different including zinc deficient mice (data not shown).

Zinc Concentrations in Serum and Skin

Zinc concentrations in serum and skin were increased in dose dependent manner. Serum zinc concentration in mice fed a zinc deficient diet was statistically reduced by 51% compared to that of mice fed a control diet (Figure 4.1). Serum zinc concentration was statistically significant in mice fed a 500 ppm zinc diet and a 1000 ppm zinc diet than mice fed a control diet.

Zinc concentration of wounded skin in mice fed a zinc deficient diet was statistically lower than those of mice fed a control diet and mice fed zinc supplemented diets (Figure 4.2). Zinc concentration in wounded skin was slightly decreased compared to its own pair unwounded skin except a control group. Although zinc concentration in unwounded skin was not statistically significant compared to that of mice fed a control diet it was statistically lower that that of mice fed a 500 ppm zinc diet.
Wound Closure

Wound closure of mice fed a 1000 ppm zinc diet was statistically significant compared to that of mice fed a control diet through day1 to day7 (Table 4.1, Figure 4.3, Figure 4.4). The rate of wound closure in mice fed a zinc deficient diet was statistically slower than that of mice fed a control diet in the early inflammatory stage (day 2- day 4). Although wound closure in mice fed a 500 ppm zinc diet was faster it was not statistically significant.

Neutrophil Infiltration

The effect of dietary zinc on neutrophil infiltration in cutaneous wounds in mice was examined (Figure 4.5). Neutrophil infiltration in mice fed a zinc deficient diet and in mice fed a 1000 ppm zinc diet was delayed compared to that of mice fed a control diet at 12 hr after wounding. However, neutrophil infiltration in mice fed a zinc deficient diet was normalized at 24 hr but not in mice fed a 1000 ppm zinc diet. Mice fed a 500 ppm zinc diet accelerated neutrophil infiltration at 6 hr and at 12 hr after wounding compared to that of mice fed a control mice.

mRNA expression of IκB

High level of IκB mRNA was detected in group fed a 500 ppm zinc diet at 12hr and day 1 when compared to that of mice fed a control diet. But, expression of IκB mRNA in mice fed either a zinc deficient diet or a zinc 1000 ppm diet was barely detected at 12 hr (Figure 4.6). Expression of IκB mRNA in mice fed either a 500 ppm
zinc diet or a control diet was little decreased compared to that at 12 hr but still highly expressed but in mice fed a 1000 ppm zinc diet was increased at day 1 as compared to that at 12 hr after wounding.

**mRNA expression of Proinflammatory cytokines**

mRNA expression of IL-1β and TNF-α was detected in the dermis along the wound edge and clot. However, the difference in gene expression of IL-1β and TNF-α was not observed (data not shown).
4.5 DISCUSSION

Nutritional status is a critical determinant of the wound healing process. In particular, zinc deficiency has been associated with delayed wound healing, and zinc supplementation has even been proposed as a preventive measure to aid wound healing. In these experiments we have demonstrated that zinc deficiency significantly delayed wound closure during the early inflammatory stage compared to control mice (p<0.05) (Table 4.1, Figure 4.3, Figure 4.4). Moreover, our results indicated that high-dose zinc supplementation (1000 ppm) also delayed wound closure during the inflammatory and proliferative stages when compared to control animals. The findings regarding wound healing in mice supplemented with 1000 ppm zinc are particularly noteworthy, and seem to indicate that at high doses zinc may exert deleterious, if not toxic, consequences. In contrast, wound closure in mice supplemented with 500 ppm zinc was comparable to that observed in control mice. Furthermore, average wound size during the early inflammatory stage in mice supplemented with 500 ppm zinc was in fact smaller than wound size in control mice, although the results were not statistically different. Taken together, these data clearly demonstrate that while moderate levels of zinc supplementation may be beneficial, excessive zinc supplementation is unequivocally deleterious in terms of its influence on cutaneous wound healing.
In addition to our observations of the effect of different levels of dietary zinc on wound healing, we also observed a strong relationship between wound tissue as well as circulating zinc levels, and wound size. As expected, wound and serum zinc levels were significantly lower, and wound size was significantly greater, in mice fed the zinc deficient diet (<0 ppm zinc) compared to mice fed the control diet (50 ppm zinc) (p<0.05) during the early inflammatory stage (Figure 4.1, Figure 4.2). Similarly, wound and serum zinc levels were significantly lower, and wound size was significantly greater, in mice fed the zinc deficient diet (<0 ppm zinc) compared to mice fed the 500 ppm zinc supplemented diet. However, neither wound zinc nor circulating zinc level was an unambiguous predictor of wound size. In particular, wound size was significantly larger in mice fed a high zinc diet (1000 ppm) compared to control mice, despite the fact that there was no statistically significant difference in wound tissue or circulating zinc levels between the two groups. Thus, it appears that the deleterious effects of dietary zinc supplementation at 1000 ppm zinc may be mediated by a mechanism that is independent of either serum or wound tissue zinc level. Indeed, it has been demonstrated in human studies that a zinc intake 20-fold greater (300 mg/day) than current RDA values (15 mg/day) decreased several indices of immune function, including the chemotactic response and phagocytic activity of neutrophils (31). Although the mechanisms underlying such observations remain unknown, it has been suggested that very high zinc intakes may decrease copper absorption and lead to copper deficiency and anemia, factors that may play an important role in the observed immunodepression in patients supplemented with high levels of zinc (32).
Neutrophils are the predominant cell type at the wound site within 24 hours of wounding where they function not only as phagocytic cells, but also as cells that synthesize and release various proinflammatory cytokines that are essential to promoting subsequent steps in the wound healing process (33). Results from our experiments indicate that consumption of either a zinc deficient (<0 ppm) or a high zinc (1000 ppm) diet may impair the wound healing process through their effects on neutrophil infiltration at the wound site (Figure 4.5). Neutrophil infiltration of wound tissue was substantially reduced during the early inflammatory stage in mice fed either the zinc deficient or high-zinc diet compared to mice fed the control diet, and this may in part account for the greater wound size observed in these treatment groups as compared to control mice. Consistent with these findings, we also observed accelerated neutrophil infiltration at the wound site in mice fed the 500 ppm zinc diet compared to control mice. This may be a significant observation, as wound size was consistently smaller in mice supplemented with 500 ppm zinc compared to controls.

Similar to our results of the effect of dietary zinc on neutrophil infiltration, the expression of IκB mRNA was greatly influenced by dietary zinc treatment (Figure 4.6). IκB mRNA expression in either the zinc deficient or the 1000 ppm zinc supplemented group was delayed, indicating that NFκB activation was low in these groups compared to that of control mice at 12 hr. Notably, expression of IκB in mice fed a 500 ppm zinc diet was higher at 12 hr and 24 hr compared to control. However, gene expression of proinflammatory cytokines (e.g. IL-1β and TNF-α) was not changed by either zinc deficiency or dietary zinc supplementation. Because wound size during the early inflammatory stage was only correlated with IκB expression but not with
proinflammatory cytokine expression, these results suggest that zinc status may not exert its selective effects on wound healing through the transcription factor NFκB.

Zinc has been utilized for the treatment of wounds for decades. In these experiments, we have investigated the effects of dietary zinc on cellular and molecular events in the early inflammatory stage of cutaneous wound healing in vivo. Our data demonstrated that both low dietary and very high dietary zinc delays wound closure, decreases neutrophil infiltration and reduces IκB expression during the early inflammatory stage of wound healing. However, a mechanism or mechanisms have not yet been elucidated that account for these observations.

We investigated that delayed wound healing observed in zinc deficiency consistent with decreased IκB expression. Zinc deficiency has been reported to reduce NFκB binding activity in vitro and in vivo (34). Inconsistent with this report, our data demonstrate that expression of TNF-α and IL-1β, other target genes of NFκB activation, was not significantly changed in wound tissue of zinc deficient mice. NFκB is a redox sensitive transcription factor that modulates immune genes such as cytokines, chemokines, cell adhesion molecules, and extracellular matrix molecules, each being critical to the wound healing process (1). In addition, TNF-α and IL-1β have been known to activate NFκB binding activity through the amplification loop.

Our observations that mice fed a high dose zinc diet (1000 ppm) exhibited similar characteristics as mice fed a zinc deficient diet (i.e. delayed wound healing, decreased neutrophil infiltration, and decreased IκB activation) at first seems paradoxical.
However, it should be noted that zinc has been shown to have variable effects on NFκB activity. For example, zinc is required for NFκB binding to DNA in vitro (35). However, zinc has also been shown to inhibit NFκB binding in vitro (36) and in vivo when supplemented at 20 times the normal level (37). In addition, high dose zinc supplementation may cause copper deficiency which can cause both neutropenia and functional impairment of neutrophils (38). Therefore, NFκB activation may have differential regulatory effects on downstream genes because selectively regulated mRNA expression of a downstream gene was observed in either zinc deficiency or high dose zinc supplementation.
4.6 ACKNOWLEDGMENTS

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Figure 4.1 Serum zinc concentration in mice fed different levels of zinc diet
Female CD-1 mice (n=20, 3 weeks of age) were randomized into 4 groups. Each group was fed a zinc deficient (0 ppm), a control (50 ppm), a 500 ppm or 1000 ppm zinc diet for 2 weeks. Serum zinc level was measured at the second week of diet treatment. The values are means ± S.E. with significance at p < 0.05.
Figure 4.2 Skin zinc concentration in mice fed different levels of zinc diet
Female CD-1 mice (n=16, 3 weeks of age) were randomized. Each group was fed a zinc deficient (0 ppm), a control (50 ppm), a 500 ppm or a 1000 ppm zinc diet for 2 weeks. Zinc levels in unwounded skin were measured at the second week of diet treatment and those of wounded skin were measured at day 1 after wounding. The values are means ± S.E. with significance at p < 0.05.
Table 4.1 Wound closure during cutaneous wound healing in mice fed different levels of zinc diet
Female CD-1 mice (n=24, 3 weeks of age) were randomized into 4 groups. Each group was fed a Zn deficient (0 ppm), a control (50 ppm), a 500 ppm or a 1000 ppm zinc diet for 2 weeks. Wound size was statistically evaluated by one-way ANOVA. The values are expressed means ± S.E. with different superscript letters (a, b and c) at p < 0.05. Ratio of wound size was calculated by wound size/ initial wound size. a, b and c show statistically significant among groups
Female CD-1 mice (n=24, 3 weeks of age) were randomized into 4 groups. Each group was fed a Zn deficient (0 ppm), a control (50 ppm), a 500 ppm or a 1000 ppm zinc diet for 2 weeks. Wound size was statistically evaluated by one-way ANOVA. The values are expressed means ± S.E. with different superscript letters (a, b and c) at p < 0.05. Ratio of wound size was calculated by wound size/ initial wound size. a and b show statistically significant among groups.
Figure 4.4 Wound closure during the early stage of cutaneous wound healing in mice fed different levels of zinc

Female CD-1 mice (n=18, 3 weeks of age) were randomized into 4 groups. Each group was fed a zinc deficient (0 ppm), a control (50 ppm), a 500 ppm or a 1000 ppm zinc diet for 2 weeks. Wound size was statistically evaluated by one-way ANOVA. The values are expressed means ± S.E. with different superscript letters (a, b and c) at p < 0.05. Ratio of wound size was calculated by wound size/ initial wound size. a and b show statistically significant among groups.
Figure 4.5 Neutrophil infiltration in wound during the early stage of cutaneous wound healing in mice fed different levels of zinc diet
A: zinc deficient mice; B: control mice; C: mice fed a 500 ppm zinc diet; D: mice fed a 1000 ppm zinc diet; -1: at 6 hr; -2: 12 hr; -3: 24 hr
Arrows indicate neutrophil infiltration in wounds
Figure 4.6 IκBα expression in wound during the early stage of cutaneous wound healing in mice different levels of zinc diet (*In Situ* Hybridization)

A: zinc deficient mice; B: control mice; C: mice fed a 500 ppm zinc diet; D: mice fed a 1000 ppm zinc diet; -1: at 12 hr; -2: 24 hr; -3: 48 hr

Fluorescence density indicates the amount of IκBα expression in wounds
4.7 REFERENCE


Wound healing is a cascade of processes associated with inflammatory cells, immune factors, various enzymes and nutrients. Notably, protein energy malnutrition and zinc deficiency are well-known worldwide health problems which cause decreased immune function and delayed wound healing. However, the effect of nutritional status, particularly PEM and zinc deficiency, on the early inflammatory stage of cutaneous wound healing remains largely unknown. Therefore, an examination of the effects of malnutrition and nutritional supplementation on the early inflammatory stage of wound healing may provide some key insights not only into the wound healing process, but also into strategies that may provide significant clinical benefit.

Results from our experiments demonstrated that PEM and zinc deficiency delayed wound healing. Specifically, we observed decreased and delayed neutrophil infiltration and gene expression of proinflammatory cytokines coincident with decreased NFκB activation. However, nutritional supplementation exhibited beneficial effects on the early stage of wound healing. Specifically, N-acetyl cysteine (NAC) has been known to
protect tissue damage by ROS and to enhance immune defense as a glutathione precursor. Zinc as well as NAC has also been known to prevent tissue damage from ROS and to increase immune function. In our experiments, NAC supplementation in PEM restored neutrophil infiltration, gene expression of IL-1β and TNF-α through increased NFκB activation during the early inflammatory stage. Further, we demonstrated that zinc supplementation with 500ppm zinc enhanced neutrophil infiltration and accelerated NFκB activation and wound closure. However, high zinc supplementation (1000ppm) showed adverse effect in neutrophil infiltration and NFκB activation as zinc deficiency showed in the early stage of wound healing.

The other significant finding from our studies concerned the role of ROS in wound healing using CuZnSOD transgenic (over-expressor and knockout) mice. Wound closure was delayed in CuZnSOD over-expressor mice fed a control diet but was accelerated in the same transgenic mice fed a protein deficient diet. In addition, ROS was essential in the early stage of wound healing but excessive ROS was detrimental in later stages in healthy subjects. However, excessive ROS caused delayed wound healing in antioxidant enzyme defected PEM mice during the early inflammatory stage.

In summary, this body of work has demonstrated that although PEM delayed wound healing during the early inflammatory stage, NAC supplementation could partially restore the early healing process. We also demonstrated that zinc deficiency caused decreased immune response and delayed wound healing, but that moderate zinc supplementation, in contrast with the deleterious effects of megadoses of zinc (20 times of control), can accelerate wound healing by enhancing immune function. These findings suggest that effective intervention strategies can be employed for malnourished patients.
that may be at risk for delayed wound healing, as well as for high-risk individuals presenting with chronic diseases.


