EFFECT OF DIETARY ALUMINUM ON IRON AND FERRITIN METABOLISM

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI'I IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

NUTRITIONAL SCIENCES

DECEMBER 2004

By
Joyce KR Wu

Thesis Committee:

Michael A. Dunn, Chairperson
Yong Soo Kim
C Alan Titchenal

ACKNOWLEDGEMENTS

My thesis and master studies could not be completed without the help and time of several special people. I am sincerely grateful for their support and encouragement.

My heart-felt thanks goes to my advisor Dr. Michael Dunn for his guidance and for giving me the opportunity to work in his laboratory. Also I want to thank him for being so patient with me through my thesis project and master studies.

Also my sincere gratitude goes to Dr. Titchenal for his encouragement; Dr. Yong Soo Kim for allowing me to use his equipment; Mr. Steve Speilman for his support and encouragement; Mr. Wayne Toma and Mr. Min Lim for their generous help in computers; and to Soccoro Tauyan for her help in animal care, as well as her generosity and kindness.

Lastly, I'm grateful for the unconditional love and support from my family. I am blessed to have such a wonderful family.

ABSTRACT

Aluminum (Al) toxicity is known to cause microcytic anemia, osteomalacia, and encephalopathy, but the mechanisms are poorly understood. Although Al is a neurotoxicant, its role in Alzheimer's disease remains controversial. One hypothesis for the toxicity of Al is that it disrupts cellular iron (Fe) homeostasis, causing iron to become cytotoxic. Cell culture studies have shown that Al suppresses the synthesis of ferritin, inhibits iron uptake into this important Fe sequestering protein, and increases oxidative damage. No studies have been done to investigate if these results occur in vivo. The objectives of this study were to determine if dietary Al alters the regulation of tissue iron and ferritin levels and inhibits iron sequestration by ferritin in an animal model. Because the kidney accumulates Al faster than the brain, kidneys were studied as a model tissue. Groups of day-old chicks were fed diets containing one of three levels of Fe (control, intermediate, or high Fe) with and without added Al. After 4-weeks, the blood, liver and kidneys were removed. Kidneys were fractioned into debris pellet, mitochondrial pellet, and supernatant (cytosol) by differential centrifugation. The Fe concentrations in tissues and in each subcellular fraction were measured by ICP emission spectroscopy. Ferritin protein and ferritin-bound iron in kidney cytosol were measured by native-PAGE. All data were analyzed by ANOVA and subsequently, analysis of covariance was used to examine the effect of Al on ferritin protein, ferritin bound iron and iron in subcellular fractions independent of effects due to changes in kidney Fe concentration. Results from ANOVA showed that kidney, liver, and serum Fe concentrations were lower in the Al treated group; this indicated that Al inhibited Fe absorption. Analysis of covariance indicated that Al increased the Fe concentration in the debris pellet, but had no effect on

mitochondrial and supernatant iron. Regression of kidney Fe concentration versus kidney ferritin protein levels showed that Al did not inhibit the upregulation of ferritin by Fe. However, high dietary Al strongly inhibited iron uptake by kidney ferritin. These results confirm that Al disrupts iron homeostasis and suggests that Al-loaded animals have higher concentrations of reactive free iron in tissues. Potentially, this may increase the risk of Fe-induced oxidative stress.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

ALS	Amyotrophic Lateral Sclerosis
Fe	Iron
Fe^{+2}	Ferrous Ion
Fe^{+3}	Ferric Ion
DMT 1	Divalent Metal Transporter 1
FPN 1	Ferroportin 1
IREG 1	Iron-Regulated Transporter 1
MTP 1	Metal Transport Protein 1
Apo-Tf	Iron-Free Transferrin
$(FeOOH)_8(FeO)(PO_3H_2)$	Ferric hydroxyl phosphate polymer
MELL	Mouse Erythroleukemic Cell Line
IRE-BP	Iron Regulatory Element-Binding Protein
IRP	Iron Regulatory Protein
IRE	Iron Regulatory Element
TfR	Transferrin Receptor
UTR	Untranslated Regions
4Fe-4S	Iron Sulfur Cluster
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
$O2^{\cdot}$	Superoxide radical
$OH^{\cdot}$	Hydroxyl radical
O2	Dioxygen
H_2O_2	Hydrogen peroxide
LDL	Low-Density Lipoprotein
Al	Aluminum
FEC	Friend Erythroleukemia Cells

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Iron toxicity normally does not pose a serious health hazard to the general population because iron is not well absorbed into the body. However, when iron toxicity does occur it could lead to detrimental or fatal consequences. Excess iron in cells can result in acute myocardial infarction, atherosclerotic lesions and certain types of cancer such as liver, renal, colorectal, lung, and stomach cancer (Candore et al. 2003, Tuomainen et al. 2003, Waalen et al. 2002, Gaenzer et al. 2002, Lemmer et al. 1999, Fracanzani et al. 2001, Ali et al. 2003, Stevens et al. 1988, Nelson et al. 2001, Chen et al. 1990, Pham et al. 1992, and Xu et al. 1996). Accumulation of iron in the brain is associated with Parkinson's disease and Alzheimer's disease (Youdim et al. 1993, Harley et al. 1993, and Bartzokis 2004). Increased levels of cellular free iron is correlated with increased of oxidative damage in the cell (Harley et al. 1993, Jang and Surh 2002, Youdim et al. 1993, and Walter et al. 2002). Iron toxicity occurs when iron homeostasis is disturbed by genetics, excessive intakes, disease processes or some agent such as aluminum.

Aluminum is extensively used in modern-day life. The sources of human intake of aluminum include food, water, pharmaceuticals, and environmental exposure. Despite the abundance of aluminum in the environment and its presence in biological systems, it has no known biological function. In fact, the accumulation of aluminum has been linked

to neurological disorders such as dialysis dementia, Alzheimer's disease and ALS Parkinsonism dementia complex (Borenstein et al. 1990, Broe et al. 1990, Jansson 2001, and Pratico et al. 2002). Aluminum toxicity also causes microcytic anemia, osteomalacia, and neurological damage (Abreo and Glass 1993). However, the mechanism leading to the toxicological consequences are poorly understood. Several studies suggest that one way aluminum potentiates its toxic effect is by altering iron metabolism and cellular iron homeostasis (Bondy and Kirstein, 1996 and Perez et al. 2001). One hypothesis is that aluminum can increase the synthesis of transferrin receptors while suppressing the synthesis of the iron storage protein ferritin. This results in the increase of intracellular "free" or reactive Fe that can be cytotoxic by inducing oxidative stress.

In the following literature review, I will not discuss all of the many proposed mechanisms for how aluminum can induce neurological disorders because they are outside the scope of this thesis (reviewed by Exley, 2001). But, I will discuss mechanisms related to aluminum's effect on iron metabolism with an emphasis on the regulation of ferritin, transferrin receptors, and intracellular iron homeostasis.

PART 1 IRON METABOLISM

SOURCES OF IRON

Iron in the diet consists of heme and nonheme iron (iron not bound to heme). Heme iron makes up 10 percent of the iron in the average American diet and is found primarily in animal flesh. The other 90 percent is nonheme iron, which comes from plants, meat, and diary products.

BIOVAILABILITY

The absorption of heme iron is twice as efficient as nonheme iron because it is absorbed as an intact heme molecule and is not affected by inhibitory ligands or chelators in the diet. Heme iron absorption is influenced by body iron status. Absorption may range from 15% with normal iron stores to 35% in a deficiency state (Groff and Gropper 2000).

The bioavailability of nonheme iron varies from 1% for rice and spinach, 3% for maize and black beans, 4% for lettuce, 5% for wheat, to 7% for soybeans (Layrisse 1975). Inhibiting ligands and the low solubility of non-heme iron reduce its bioavailability. Diets including protein factors along with vitamin C may enhance the absorption of nonheme iron. Non-heme iron absorption is also increased in iron deficiency.

IRON ABSORPTION MECHANISM

Heme Absorption

Heme iron is absorbed along the small intestine, particularly in the duodenum and jejunum. Little is known about the mechanism of transport of heme iron. It has been suggested that heme iron is transported across the mucosal brush border as an intact molecule via a heme transporter/receptor on the apical surface of duodenal absorptive cells (Uc et al. 2004). After it enters the cell, heme is degraded by heme oxygenase-1 and the iron is released. The released heme iron and nonheme iron are metabolized similarly in the enterocytes as discussed below.

Nonheme Iron Absorption

How nonheme iron crosses from the small intestine into the enterocytes and into the blood is not well understood. Researchers have identified a divalent metal transport protein 1 (DMT1; also known as DCT1 or Nramp 2) on the apical membrane and an iron transport protein (ferroportin 1) on the basolateral surfaces of the mucosal cell (Ke et al. 2003, Le and Richardson 2002, Abboud and Haile 2000, Mckie et al. 2000, and Conrad et al. 2000). Nonheme iron exists as ferrous (Fe^{2+}) or ferric (Fe^{3+}) ions in food. Hydroc

hloric acid and pepsin from gastric secretions hydrolyze the food components and release the nonheme iron. The high H+ concentrations in the stomach convert the iron to the ferric ion. As the Fe^{3+} ion reaches the small intestine a proportion of it will be reduced back to Fe^{2+}, the more soluble form of iron, by the enzyme ferric reductase located on the apical (luminal) enterocytes membrane. The ion then enters the cell through the DMT1 transporter (Hentze et al. 2004). DMT1 has also been identified on

brain (Jeong and David 2003) and kidney cell membranes (Ferguson et al. 2001 and Hubert & Hentze 2002).

Reducing agents such as ascorbic acids and chelators such as citric acid, lactic acid, malic acid, tartaric acid, and the acidic amino acids can help increase dietary iron absorption by keeping the iron in a soluble form so the iron can enter through DMT1. A diet high in these agents can enhance nonheme iron absorption (Guthrie and Picciano 1995). However, if the iron forms insoluble aggregates in the small intestine the ion will be excreted in the feces along with other undigested food components. Low gastric acid, high dietary fiber, high phosphorus intakes, certain proteins (milk, cheese, and eggs), polyphenols (tannins in coffee and tea), phytates (whole grains, brans, and soy products), and oxalates (spinach, rhubarb, and chocolate) can decrease the bioavailability of iron. Therefore, the absorption of iron from eggs, milk, and spinach is less efficient because of the inhibitory ligands present in these foods that inhibit the absorption of iron into the mucosal cells. Calcium and zinc may also inhibit iron absorption, but the mechanisms are not well understood.

Heme and Nonheme Iron Metabolism in the Enterocytes

Once the iron (heme or nonheme) is absorbed into the enterocytes, part of the absorbed iron (in the Fe^{2+} form) can be use for normal cellular function. Depending on the body iron status, a varied amount of iron will translocate across the basolateral membrane of the enterocytes into the blood via ferroportin. Several studies suggested that ferroportin 1 (also called Fe-regulated transporter 1 (IREG 1) or metal transport protein 1 (MTP 1) is the molecule involved in the export of iron into the circulation

(Hentze et al. 2004, Le and Richardson 2002, and Abboud and Haile 2000). After iron enters the circulation it is oxidized to Fe^{3+} by ceruloplasmin, a copper containing plasma protein, or by hephaestin, a membrane-bound multicopper containing ferroxidase. Oxidized Fe^{3+} is quickly loaded onto transferrin, the iron transport protein in the plasma. Iron not transported into the blood remains in the enterocytes bound to ferritin, the iron storage protein. Iron trapped in ferritin is eliminated along with the sloughing of the old mucosal cells. These intestinal cells provide a barrier to help regulate the level of iron absorbed from diets to prevent the body from iron overload.

Regulation of Intestinal Iron Absorption

Absorbed iron is not easily eliminated from the body. Because of this reason, controlling the absorption of iron is crucial to prevent iron overloads. A proposed model by Nicolas et al. (2001) suggests that the iron regulatory peptide, hepcidin, produced in the liver, appears to modulate intestinal iron absorption. This model proposes that increased iron uptake by the liver increases the synthesis and secretion of hepcidin. During high iron status, the number of transferrin-transferrin receptors complexes are increased in the liver. This stimulates the production and release of hepcidin by the liver into the plasma. Hepcidin modulates intestinal iron absorption by modifying the crypt cells to produce a low level of duodenal ferric reductase expression and iron transport proteins [(Ferroportin 1 (FPN1), and divalent metal transport protein 1 (DMT1)] (Leong and Lonnerdal 2004). When the old enterocytes are sloughed off, these new crypt cells differentiate into mature enterocytes, resulting in decreased amounts of ferric reductase enzymes and iron transport proteins. As a result, there is a decrease in dietary iron

absorption (Leong and Lonnerdal 2004). Leong and Lonnerdal's (2004) observation agrees with Nicolas's (2001) model in that they observed an inverse correlation between hepcidin, duodenal iron transporter expression, and iron absorption.

DISTRIBUTION OF IRON IN THE BODY

Transferrin delivers iron in the plasma to tissues for use or storage. Transferrin is a single polypeptide chain around 80 kDaltons with two iron-binding sites on both ends of the protein (Crichton 1991). Each transferrin can hold up to two ferric ions. The major function of transferrin is to transport extracellular iron into the cell and to maintain plasma iron in a soluble non-reactive form that is suitable for cellular uptake. It acts almost like an antioxidant that houses the iron in a safe form to prevent iron-induced oxidative stress in the circulation.

A large quantity of the plasma iron is taken up by bone marrow for hemoglobin synthesis and smaller quantities are taken up by other tissues for use in myoglobin or iron-dependent enzymes. The excess iron in cells is stored in the proteins ferritin and/or hemosiderin, primarily found in liver, bone marrow, and spleen.

INTRACELLULAR IRON METABOLISM

Cellular iron uptake and metabolism requires multiple steps. First, the iron containing transferrin molecule in the plasma binds to the transferrin receptor molecule on the tissue cell surface, the complex is internalized into the cell by receptor-mediated endocytosis. Then, the endocytotic vesicle fuses with lysosomes and matures into a proton-pumping endosome. When the interior of the endosome reaches a pH of 5.5, the

transferrin-transferrin receptor complex dissociates, the transferrin receptor is released back to the cell surface and the Fe^{3+} ions are released from transferrin. The iron-free transferrin (Apo-Tf) is recycled back into the circulation (Crichton et al. 2002). Ferric ion is reduce to ferrous ion by ferrireductase prior to its transport out of the endosome by the transmembrane iron transporter, divalent metal transporter (DMT1)/Nramp2, and translocates into the cytosol (Crichton et al. 2002). The new, cytosolic, free Fe^{2+} can enter the available iron pool for use by the cell. The excess free iron within the cell can be stored in ferritin.

FERRITIN

Structure

Ferritin, the iron sequestering and storage protein, is synthesized in most tissues, especially those with high iron contents such as liver, bone marrow, and spleen (Crichton 1971). Ferritin consists of 24 protein subunits forming a hollow core with a molecular weight around 450,000 Da (Groff and Gropper 2000). Each ferritin molecule can hold up to 4,500 iron atoms within its core (Groff and Gropper 2000). Ferrous iron enters through one of eight channels in the ferritin molecule. Ferroxidase enzymes are located along the ferritin pores to oxidize Fe^{2+} to ferric oxyhydroxide crystals or ferrihydrite, the stored form of iron within the ferritin core (Orino et al. 2004).

Ferritin Subunits

The subunit composition of ferritin varies from tissue to tissue within the same species. Ferritin contains two different subunits, the relative proportions of which vary

with tissue type. The H-subunit is a 22-kDa protein which predominates in heart ferritin. The L-subunit is a 20-kDa protein which predominates in liver and spleen. The H-subunit is 50% identical to the L-subunit and both have similar structures. The composition of subunits may influence the functional properties of ferritin (Jacob 1975). The H-subunit contains the enzyme ferroxidase to convert ferrous ions into ferric hydroxides. The L-subunit lacks this ferroxidase activity and is thought to play a role in nucleation and mineralization of the iron core. Ferroxidase in H-subunits has a central role in regulating free iron availability in the cell and, therefore, resistance to iron induced oxidative damage (Arosio and Levi 2002). A large increase of H-subunits results in an iron-deficient phenotype in mouse erythroleukemic (MELL) cell line and a resistance to oxidative stress (Picard et al. 1998). High amounts of L-subunits have no effect on iron metabolism (Corsi et al. 2002).

Ferritin Synthesis

Ferritin expression is regulated at the level of transcription and translation. The H-subunits are regulated at the translational level and the L-subunits at the transcriptional and translational levels.

The regulation of ferritin synthesis at the transcriptional level is not well understood. The expression of ferritin at the translational level is controlled by the level of cytosolic iron. Under low intracellular iron conditions, Iron Regulatory Element-Binding Proteins (IRPs) are activated and bind to the Iron-Regulatory Elements (IRE) at the 5' ends of the ferritin mRNA. The interaction of IRPs and IRE inhibits initiation of the translation of ferritin mRNA. On the other hand, under high iron levels, IRPs are

inactivated [become aconitase (IRP 1) or are degraded (IRP 2)], thus allowing the initiation of ferritin mRNA translation to continue. By controlling the availability of IRPs, cytosolic iron directly influences the synthesis of ferritin, an important molecule crucial in maintaining cellular Fe homeostasis. The importance of ferritin is made clear by the fact that ferritin knockout mice die during embryonic development.

CELLULAR IRON HOMEOSTASIS

Cellular iron homeostasis is regulated by balancing iron uptake by the cell, via the transferrin receptor, with intracellular storage and utilization so that excessive "free" or reactive iron does not accumulate. This balance is achieved by the coordinated translation of transferrin receptor (TfR) and ferritin mRNAs. The mRNAs coding for ferritin and the transferrin receptor contain similar stem-loop structures, IREs, on the 5' and 3' untranslated regions (UTR), respectively. IRPs (as mentioned above) are cytosolic RNA binding proteins that bind to IRE and control the translation or stability of the mRNA. During low intracellular iron, IRP binds to IRE on the transferrin receptor mRNA at its 3' UTR. This prevents access of endonucleolytic enzymes and protects the mRNA from degradation. The result is increased TfR expression. The increase of TfRs on cell membrane leads to increased iron uptake. Ferritin mRNA behaves differently when IRP binds to IRE. When the IRP molecule binds to the IRE on the 5' end of ferritin mRNA, it blocks the initiation factor complex (factor-4F) from binding to the 5' end of ferritin mRNA, and inhibits the translation of the ferritin message. Less ferritin means less Fe is sequestered and more is available for use in the cell.

As the cytosolic iron concentration increases, brought forth by TfRs as described earlier, the two forms of IRPs (IRP1 & IRP2) behave differently as they come in contact with the cytosolic iron. The binding of cytosolic Fe to IRP1 forms a Fe-Sulfur cluster (4Fe-4S) and converts IRPs into cytosolic aconitase in the TCA cycle. This prevents IRP1 binding to IRE on TfR or ferritin mRNA (Roy et al. 2002). Conversely, the binding of iron to the cysteines-sulfur cluster region referred to as the "iron-dependent degradation domain" on IRP2, leads to oxidative modification at that site and degradation of the IRP2 protein (Bourdon et al. 2003). It was hypothesized that the cysteines could ligate free iron which then mediates oxidation of IRP2 creating a recognition signal for the protein ubiquitin that signals proteases to degrade the IRP2 protein (Bourdon et al. 2003, and Yamanaka et al. 1999). The degradation of IRP2 or the conversion of IRP1 into aconitase prevents the binding of IRPs with IRE on mRNA. In the case of TfR mRNA, this allows the endonucleolytic enzymes to bind to the 3' UTR, and induce transferrin receptor mRNA degradation. As a result, this leads to decreased TfR expression. On the other hand, the 5' end of ferritin mRNA translocates to the ribosome and initiates the translation of the ferritin message; therefore, there is an increase in ferritin concentration to store the free iron.

Normally, the body is able limit the amount of reactive free Fe via regulating Fe absorption by the enterocytes and, at the cellular level, by regulating the transferrin receptor for cellular Fe uptake, and ferritin level for iron storage. However, sometimes genetics, high iron intake, or high exposure to chemicals from the diet such as aluminum can disrupt the normal function of these iron homeostasis molecules; as a result this can lead to iron toxicity.

IRON TOXICITY

The toxicity of iron is well established both clinically and experimentally. Symptoms of moderate cases of iron overload can range from nausea to diarrhea to constipation. However, chronic iron toxicity has been correlated to certain forms of cancer and atherosclerosis. Many studies also have linked iron as a potential culprit for neurological disorders such as Alzheimer's and Parkinson's disease.

Results from clinical and epidemiologic studies demonstrated strong associations between excess iron and the development of cancer (Deugnier 2003). Bhasin et al. (2003) observed increased tumor response in mice when iron levels of 0.5, 1.0, and 1.5mg/mouse were injected once a week at the promotion stage of skin carcinogenesis. Also, Adzersen et al. (2003) found an increased risk of lung cancer and other cancers of the upper aero-digestive tract among foundry workers.

The best example to illustrate the effect of iron overload is hereditary hemochromatosis. Hemochromatosis is a common, autosomal recessive genetic disorder affecting approximately 1 in 200 individuals of Northern European ancestry (Davis and Bartfay 2004). It is characterized by an increase in iron absorption and results in the accumulation of iron in cells of the liver, pancreas, heart, and other organs (Davis and Bartfay 2004). The accumulation of iron can cause organ injury, particularly to the liver, heart, and endocrine pancreas (especially insulin-secreting β-cells) (Toyokuni 2002). A study conducted in Denmark indicated that the major causes of death in hereditary hemochromatosis are hepatic failure with cirrhosis and hepatocellular carcinoma (Milamn et al. 2001). Three studies concluded that hemochromatosis patients have

greater risk for hepatocellular carcinoma than in the age-matched control population (Niederau et al. 1985, Bradbear et al. 1985, and Hsing et al. 1995). Shaheen et al. (2003) suggested that HFE gene mutations (the gene responsible for hereditary hemochromatosis) are associated with an increased risk of colon cancer. Furthermore, when HFE mutations coexist with mutations in the transferrin gene, the risk for developing Alzheimer's disease increased; however, this concept is still controversial (Robson et al. 2004).

Hemochromatosis cases have also been linked to heart disease. Three prospective studies have observed an increased risk of cardiovascular disease incidence and deaths among hemochromatosis subjects (Tuomainen et al. 1999, Rasmussen et al. 2001, and Roest et al. 1999). However, other studies did not find any significant associations between hemochromatosis populations and cardiovascular disease (Calado et al. 2000, Hetet et al. 2001, and Franco et al. 1998).

The mechanism behind the devastating effect of excess iron is not well understood. Researchers suggested iron is capable of mediating tissue damage by promoting the production of reactive oxygen species (ROS) and free radicals in the body (Harley et al. 1993, Jang and Surh 2002, Toyokuni 2002, Davis and Bartfay 2004, and Cederbaum 2003). In normal healthy individuals, the levels of ROS in the body are minimized by antioxidants (e.g., SOD, glutathione, vitamin E) to prevent the induction of oxidative stress. However, iron overload can aggravate the level of free radicals (e.g., $O_2^{\bullet -}$ and $OH^{\bullet}$) via the Fenton/Haber-Weiss reactions and increase the susceptibility of cellular membranes, proteins, and DNA to attacks by ROS.

Redox cycling is a characteristic of iron, which underlies its toxicity (Aisen et al. 2001). The one-electron reduction of dioxygen (O_2) by Fe^{2+} results in superoxide formation ($O_2^{\bullet -}$) which subsequently dismutates to hydrogen peroxide. The reaction further proceeds to hydroxyl radical formation induced by the Fenton reaction:

$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -}$

$Fe^{2+} + O_2^{\bullet -} + 2H^+ \rightarrow Fe^{3+} + H_2O_2$

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$ (Fenton reaction)

The hydroxyl radical is possibly the most powerful oxidant. It will attack proteins, nucleic acids and carbohydrates, and initiate chain-propagating lipid peroxidations (Aisen et al. 2001). Whittaker and Chanderbhan (2001) observed that an increase in non-heme iron in liver from 18 to 3500 μg/g correlated with an increase in lipid peroxidation, increase in serum phospholipids, and a change in the ratios of saturated to unsaturated fatty acids. Studies indicated that iron loading results in an increase in malonaldehyde production, a decrease in antioxidant levels such as gluthione, a reduction of specific activity of complex I and IV of the respiratory chain in Parkinson's disease, and stimulation of lipid peroxidation in Parkinson's patients (Youdim et al. 1993 and Harley et al. 1993).

Other studies suggested oxidative stress induced by ROS as the pathology of Alzheimer's disease (Jang and Surh 2002 and Ng 2004). Jang and Surh (2002) suggested that the production of ROS leads to beta-amyloid induced apoptotic cell death.

During the past decade, many studies have provided evidence to support the role of oxidative stress in the development of cardiovascular diseases (Lieu et al. 2001, Emerit et al. 2001, Chau 2000, and Martinet et al. 2002). It is hypothesized that the oxidation of

low-density lipoprotein (LDL) and lipid, induced by iron, is involved in the process of plaque formation seen in atherosclerosis (Gurzau et al. 2003). Martinet et al. (2002) observed elevated levels of oxidative DNA damage in human atherosclerotic plaques. However, other studies found no association between the role of body iron stores and cardiovascular disease risk (Moore et al. 1995 and Iribarren et al. 1998).

CONCLUSIONS

Maintaining cellular iron homeostasis is crucial to our daily lives. The disruption of iron homeostasis can pose serious health problems and sometimes even death. Elevated cellular iron concentrations can induce oxidative stress to the cell and are linked to the pathology of diseases such as cancer, Alzheimer's, Parkinson's and heart-related conditions (Jang and Surh 2002, Ng 2004, Harley et al. 1993, Rasmussen et al. 2001, Hetet et al. 2001, Shaheen et al. 2003, Milman et al. 2001, Adzersen et al. 2003, and Toyokumi 2002). Evidence suggests that iron overload results in increased lipid peroxidation, DNA lesions, and apoptosis induced by ROS via the Fenton reaction. As a result, it is critical that the body regulates iron absorption and cellular iron homeostasis.

In the normal individual, the concentration of free cellular iron is tightly monitored by IRPs that regulate transferrin receptors and the iron storage protein, ferritin. However, a small amount of people with hemochromatosis, or overdose on iron supplements can create difficulties in maintaining iron homeostasis. Recent studies have proposed that aluminum is an agent that may also alter iron homeostasis. One of the proposed hypotheses is that aluminum can decrease the synthesis of ferritin and increase expression of transferrin receptors. The disruption of the normal synthesis of transferrin

receptors and ferritin could create increased amounts of reactive iron residing in the cell, and result in an increase of oxidative stress induced by the Fenton reaction (Abreo et al. 1994 and Yamanaka et al. 1999).

PART II ALUMINUM TOXICITY AND ITS EFFECT ON IRON METABOLISM

SOURCES OF ALUMINUM

Food, water, and pharmaceuticals are the most common sources of aluminum exposure (Greger and Sutherland 1997). According to the 1984 U.S. Food and Drug Administration Total Diet Study, the average daily intakes of aluminum for adult (25-30 years old) males and females were 13.8 and 8.7mg Al/day, respectively (Greger and Sutherland 1997). Aside from food and water, there are other important sources such as pharmaceutical, environmental and occupational exposures.

Dietary Exposure

Aluminum is widely distributed in foods such as processed cheeses, baking powders, cake mixes, frozen dough, pancake mixes, soy-based milk products, and food additives (Nayak 2002). Aluminum can be absorbed by produce grown in soil that has a pH lower than 4.5-5.0 (Nayak 2002). The concentration of aluminum in tea leaves ranges from 1mg Al/g up to 17mg Al/g depending on the geographical location where it was grown (Eden 1976). Leaching of aluminum from beverage cans and cook-wares can

also increase the amount of aluminum in the diet. The average concentration of aluminum in canned soda is 0.1mg Al/g (Nayak 2002).

Medication/Pharmaceutical Exposure

Large oral doses of aluminum-containing phosphate binders, antacids, high aluminum dialysates, and calcium supplements based on oyster shells can increase aluminum intake (Greger and Sutherland 1997 and Nayak 2002). Calcium supplements based on oyster shells could contain 0.2% to 0.6% of aluminum (Bourgoin 1992). The amount of Al in enteral nutrition formulas and parental solutions ranges from 87.6 to 961.2 ng Al/ml and from 58.4 to 1232 ng Al/ml, respectively (Nayak 2002).

Aluminum is present in vaccine products such as diphtheria, tetanus, hepatitis, rabies, and anthrax (Nayak 2002). Nonprescription drugs such as antacids, buffered aspirins, antidiarrheal products, and hemorroidal medications can also increase aluminum exposure (Nayak 2002). Some individuals can consume as much as an additional 5 g of aluminum daily from antacid products (Greger 1993 and Domingo 1993).

Drinking Water Exposure

Aluminum concentrations in natural water are normally small, but higher concentrations are found in urban areas due to the increase of aluminum leached from acid rain and the use of aluminum in municipal water treatment (Nayak 2002). Several studies have found an association of aluminum in water to elevated the risk of AD and elderly cognitive impairment. However, other studies have found no effect (Rondeau et al. 2000, McLachlan et al. 1996, and Varner et al. 1998).

Certain factors may affect the absorption of aluminum in drinking water. In some studies high water pH was shown to have a large significant effect on AD rates by reducing aluminum absorption. Districts which consumed drinking water with a pH higher than 7.85 had a 50% reduction in AD risk compared to the more acidic water district (Rondeau et al. 2000). Another factor that may reduce aluminum absorption in drinking water is silicon Drinking water with more than 10ppm of silicon helps neutralize the effects of aluminum (Rondeau et al 2000 and Taylor et al. 1995).

Environmental Exposure

Aluminum makes up about 8% of the earth's crust and it occurs naturally in the soil (Nayak 2002). Higher concentrations of aluminum are found in places with coal combustion, such as aluminum mines and smelting industry. The ranges of aluminum found in the atmosphere from both natural processes and human activities are about 0.005 to 0.18mg/m^3 (Nayak 2002).

Occupational Exposure

Increased aluminum exposures are seen in groups who work in aluminum refining and metal industry, printing and publishing, auto dealerships, service stations, and places that produce metals (U.S. Public Health Services 1992). Studies reported cognitive changes, possible impairments, and other occupational hazards that are related to exposure to aluminum dusts and fumes (Nayak 2002).

GROUPS AT RISK FOR ALUMINUM TOXICITY

Renal disease patients on aluminum containing dialysis drugs are often the major population with an increased risk of aluminum toxicity. Patients with reduced renal function can sometimes ingest up to 10g Al/day from aluminum-based phosphate binders given to reduced phosphate absorption (Greger and Sutherland 1997). Any population with reduced renal function tends to accumulate aluminum over time. Infants are also at risk because they are fed with infant formulas, especially soy-based ones that may contain high levels of aluminum (Greger and Sutherland 1997). People working in aluminum refining, metal, printing, publishing, auto dealerships have increase exposure of aluminum. Also, people with acid reflux disease often increase aluminum exposure from chronic ingestion of antacids.

ABSORPTION AND TRANSPORT OF ALUMINUM

There are several locations in the body where aluminum can be absorbed; these include the gastrointestinal tract, lungs, olfactory system, and the dermal layer of the skin (Nayak 2002). The mechanism behind aluminum absorption at these sites is not well understood (Nayak 2002). Aluminum absorption through the GI tract is about 0.1% however, absorption may be increased several fold by chelators of aluminum such as citrate (Deng et al. 2000, Venturini-Soriano and Bertonn 2001, and Drueke 2002). In young normal rats, 0.05%-0.1% of ingested aluminum is absorbed in the intestine; after 2 hours half of the ingested aluminum goes mostly to the skeleton and after 48 hours the other half is excreted in the urine (Drueke 2002).

The process of how aluminum is absorbed is difficult to determine and no specific transport system has been identified. The concentration of aluminum in the GI, the solubility of aluminum, and the presence of other dietary factors affect its bioavailability (Greger and Sutherland 1997). According to Van der Voet (1992), aluminum absorption includes both paracellular passage of aluminum through tight junctions and transcellular passage involving passive, facilitated, and active transport processes through the mucosal cells. According to another study using in situ isolated duodenal segments at pH 2; approximately 23% of aluminum absorption was through nonsaturable processes and the rest was through saturable (active) processes (Greger and Sutherland 1997).

Once aluminum crosses the barrier of the mucosal cell and into the blood, it can bind to empty iron-binding sites on transferrin. McGregor et al. (1991) observed that lymphocytes and human erythroleukaemia K562 cells took up aluminum by transferrin. In vitro studies have shown that between 60% and 90% of serum aluminum is associated with transferrin (Fatemi and Moore 1991 and Soldado et al. 1997). Since aluminum and iron share binding sites on transferrin, this suggests that aluminum can follow the intracellular pathways of iron distribution.

ALUMINUM METABOLISM

The cellular uptake of aluminum-bound transferrin follows the same route as iron-bound transferrin. Transferrin receptors on the cell membrane recognize the metal-bound transferrins and the complex is engulfed into a clathrin-coated pit by endocytosis. The endocytotic vesicle then fuses with lysosomes to form an endosome. As soon as the interior environment of the endosome reaches pH 5.5 by the action of a proton pump, the

aluminum is released from transferrin and moves into the cytosol (Bali et al. 1991 and Sipe and Murphy 1991). How aluminum exits the endosome remains unknown, but iron exits via the iron transporter DMT1.

ALUMINUM DISTRIBUTION

In normal healthy individuals, the total amount of aluminum in the body is approximately 30-50mg distributed among various tissues (Nayak 2002). Another study suggests a body content of 300 mg (Skalsky and Carchman 1983). Highest concentrations of aluminum are found in the bone and the lungs (Ganrot 1986). Presumably, lungs accumulate aluminum from inhalation exposure (Nayak 2002). Aluminum contents in the bone, kidneys, muscle, heart, and brain are accumulated from oral exposure (Nayak 2002). Of these tissues, the brain has the least amount of aluminum accumulation due to the partial exclusion by the blood-brain barrier (Yokel et al. 1999). In general, the accumulation of aluminum due to oral exposure is in the order bone > liver > kidney > testes > muscle > heart > brain. Increased aluminum concentrations in the body are correlated with increased age (Nayak 2002). Aluminum accumulation in the brain is estimated at a rate of 8 μg per year (Edwardson et al. 1991). Low-level long-term exposure to aluminum may be a contributing factor in Alzheimer's disease and related neurological diseases (Yokel and McNamara 2001).

ALUMINUM TOXICITY

Aluminum has been shown to be a toxicant to the central nervous system, hematopoietic system, and skeleton in animals and humans (Yokel and McNamara 2001).

The classic example is aluminum toxicity observed in renal impaired patients consuming high levels of aluminum hydroxide as a phosphate binder. Aluminum retention and toxicity results because of decreased kidney function to eliminate aluminum (Abreo and Glass 1993). Osteomalacia, bone pain, pathological fractures, proximal myopathy, and failure to respond to vitamin D_3 therapy are features of aluminum toxicity to the skeletal system (Alfrey 1991, Kausz et al. 1999, and Rodrigues et al. 1990). Takeuchi et al. (1997) demonstrated that Al has negative effects on osteoblast function and bone formation in vivo and in vitro. In the presence of 10μM Al cultured erythroleukemia cells showed a reduction in heme synthesis (Perez et al. 2001). Aluminum toxicity has also been found to induce microcytic anemia in individuals with normal renal function and the mechanism involves inhibition of Fe incorporation into the heme molecule. Aluminum is a well known neurotoxicant, causing encephalopathy in renal failure patients, but the mechanism is unknown (Abreo and Glass 1993, Yokel and McNamara 2001, Kaiser et al. 1984, and Nasiadek and Chmielnicka 2000). Aluminum can alter the function of membranes and a variety of enzymes and proteins, but the significance of these effects to neurological disorders, Alzheimer's disease and ALS Parkinsonism dementia complex is not clear (Borenstein et al. 1990, Pratico et al. 2002, Broe et al. 1990, Jansson 2001, and Abreo and Glass 1993). Some, but not all, studies have shown an association between aluminum in drinking water and Alzheimer's disease (Rondeau et al. 2000, McLachlan et al. 1996, Varner et al. 1998, and Taylor et al. 1995). Other studies have indicated that Alzheimer's patients had higher levels of brain aluminum than age-matched controls (Yokel et al. 1999). In experimental animals studies, aluminum administered in the diet increased amyloid β plaques and neurofibrillary tangles, lesions

associated with Alzheimer's disease (Clauberg and Joshi 1993, and Van Rensburg et al. 1997). Several studies indicated aluminum has the potential to enhance iron-induced oxidative stress to the cell (i.e., lipid & protein peroxidations) (Clauberg and Joshi 1993, and Van Rensburg et al 1997). Abreo and Glass (1993) suggested that aluminum could induce lipid peroxidation by increasing the cellular iron uptake in association with decreased iron entry into ferritin. This would result in an increased "labile" iron pool and facilitate iron-induced oxidative stress via the Fenton reaction. This result can be further supported by Xie et al's. observation (1996). Xie et al. indicated that increased intraneuronal Al concentration potentiates Fe-induced oxidative stress and neuronal death.

EFFECTS OF ALUMINUM ON ERYTHROPOESIS

The effect of aluminum on iron utilization for red blood cell formation has been investigated in vivo and in vitro. However, the mechanisms are not fully understood. Human subjects exposed to moderately high serum aluminum concentrations show a decrease in the mean corpuscular hemoglobin concentration, serum ferritin and transferrin saturation (Gonzalez-Revalderia et al. 2000). Nasiadek & Chmielnicka (2000) observed a decrease in iron concentration in erythrocytes, blood, and spleen, followed by an increased in the level of free erythrocyte protoporphyrins in rats orally exposed to aluminum. The mechanisms producing anemia are thought to involve inhibition of iron incorporation into heme and/or interference with iron uptake, and utilization by immature erythrocyte (Perez et al. 2001). Aluminum-induced anemia commonly occurs with normal iron status suggesting that the toxic mechanism is at the level of iron use by the

erythrocyte. Inhibition of iron absorption by the intestine, however, may also be a factor under conditions of high aluminum intake (Cannata et al. 1991). Studies of the effects of dietary aluminum on iron absorption and tissue iron contents are contradictory, some suggesting reduced iron status, some showing no effect or increased iron in tissues (Turgut et al. 2004).

EFFECTS OF ALUMINUM ON CELLULAR IRON METABOLISM

Aluminum can modify cellular iron homeostasis by stabilizing the iron regulatory protein 2 (IRP2) (Yamanaka et al. 1999). This would promote the binding of IRP to IRE, inducing TfR expression while blocking ferritin synthesis. Yamanaka et al. (1999) demonstrated that aluminum induces the IRE binding activity of IRP2 by competitively inhibiting the oxidative modification of IRP2 by iron. This prevents ubiquitination of the IRP2 protein and proteasome degradation, leading to stabilization, and increased amounts of the protein. Stabilization of the IRP2 protein promotes TfR expression and the suppression of ferritin synthesis. This could lead to increased concentrations of intracellular free iron. Alternatively, Abreo et al. (1994) proposed that aluminum caused cytosolic iron to be partitioned into mitochondria or nuclei with the resulting reduction in cytosolic iron leading to stabilization of IRPs and suppression of ferritin synthesis. However, other studies found opposite results. San-Marina and Nicholls (1992) observed increased ferritin synthesis in animals following intraperitoneal aluminum injections. Oshiro et al. (1998) observed a decreased in the transferrin receptor mRNA level in cells pretreated with aluminum.

Although studies did not show consistent results on ferritin synthesis, several studies have shown an effect on iron uptake into ferritin. Fleming and Joshi (1991) found decreased iron loading into isolated ferritin in vitro. This study is further supported by a study using cultured Friend erythroleukemia cells (FEC). Abreo et al. (1994) demonstrated that in aluminum-loaded FEC, ferritin content was reduced as was the uptake of ^{59}Fe by ferritin. These authors suggested that the decrease of ferritin and iron uptake by ferritin can lead to an increase of reactive (labile) iron in the cell which could result in iron-induced Fenton reactions and, therefore, increase ROS production in the cell.

Aluminum's ability to promote iron-induced oxidative stress is generally accepted, but not fully understood. Practico et al. (2002) observed that dietary aluminum can enhance in vivo AD-like amyloidosis in transgenic mice bearing the Alzheimer's gene, and that this was associated with increased brain oxidative stress. Bondy and Kirstein (1996) observed that aluminum potentiates iron-induced ROS, but observed no stimulation of ROS in the presence of aluminum alone. Also, increased Fe uptake in aluminum-loaded mouse hepatocytes and Friend erythroleukemia cells showed membrane lipid peroxidations (Abreo et al. 1990 and 1991). This has led to the hypothesis that aluminum toxicity may be related to iron-induced oxidative stress.

CONCLUSIONS

Aluminum toxicity is well documented, but the mechanism of action is poorly understood. Studies demonstrated that aluminum is a potential toxicant to the central nervous system, hematopoietic system, and skeleton (Yokel and McNamara 2001, Kausz

et al. 1999, Alfrey 1991, Takeuchi et al. 1997, Perez et al. 2001, Abreo and Glass 1993, Borenstein et al. 1990, and Practico et al. 2002). Cell culture studies have provided evidence that the alteration of iron metabolism induced by aluminum may be the mechanism of aluminum toxicity (Abreo et al. 1994, Oshiro et al. 1998, Yamanaka et al. 1999, Golub et al. 1996, and Perez et al. 2001). One hypothesis is that aluminum can increase the level of reactive iron and induce oxidative stress to the cell. Aluminum has an ionic structure similar to iron and is known to bind to transferrin. This concept suggests that aluminum can bind to molecules that are intended for iron (e.g., IRP2) and could disturb their normal functions. For example, evidence indicates that aluminum can bind to IRP2 and disrupt the synthesis of ferritin and transferrin receptors; ultimately, this could lead to increased ROS. Aluminum-induced oxidative damage via excess reactive iron could result in Alzheimer's disease, as well as iron-induced diseases such as cancer and heart disease. Since most of the information on the effects of aluminum on cellular iron homeostasis have been done in cell culture, the purpose of the study described in chapter II is to test some of these hypotheses in vivo, in animals exposed to dietary aluminum.

CHAPTER 2

DIETARY ALUMINUM ALTERS IRON HOMEOSTASIS AND FERRITIN FUNCTION IN THE CHICK

INTRODUCTION

Aluminum is extensively used in modern daily life and people are getting more exposed to sources of aluminum. Sources of aluminum include food, water, and environmental and occupational exposure. Aluminum is also present in many commonly-used pharmaceuticals and cosmetics. Aluminum has no known to have no biological function despite its abundance. However, in the past few decades, the toxic impacts of aluminum on biological systems have been topics of considerable interest and debate (Exley 2001).

Neurotoxic effects of aluminum have been observed in animal studies, but the mechanism are poorly understood (Bondy and Kirstein 1996, Oteiza et al. 1993, and Pratico et al. 2002). Other studies suggest that aluminum may be the cause of neurological disorders such as Alzheimer's disease and ALS Parkinsonism dementia complex (Crichton 2001, Whittaker and Chaderbhan 2001, Shaheen et al. 2003, and Adzersen et al. 2003). In renal failure patients, a dementia similar to Alzheimer's disease has been clearly linked to accumulation of aluminum in the brain (Alfrey et al. 1976). Aluminum toxicity has also been shown to cause microcytic anemia and osteomalacia (Nayak 2002). Despite a large body of literature on the pathological effects of aluminum, the underlying causes of tissue damage remain largely unresolved.

One hypothesis for the neurotoxicity of Al is that Al indirectly promotes its toxic effect by altering iron metabolism (Abreo and Glass 1993, and D'Haese and DeBroe 2001). Early experimental studies indicated that aluminum has the potential to cause iron-induced oxidative stress leading to cellular damage (Bondy and Kirstein 1996, and Oteiza et al 1993). One proposed mechanism is that aluminum can disturb cellular iron homeostasis by increasing the translation of transferrin receptors and decreasing the translation of ferritin. The increase in transferrin receptors would lead to an increase in iron uptake into the cell; however, at the same time, a decrease in ferritin synthesis would limit the ability to store the excess iron. Therefore, this would lead to an increase in intracellular free (reactive) iron not stored in ferritin, which could cause oxidative stress and cell damage (D'Haese and De Broe 2001, and Abreo et al. 1999).

There are two current hypotheses for how Al could alter the expression of iron responsive proteins such as ferritin and the transferrin receptor. Yamanaka et al. (1999) reported that aluminum stabilized the iron regulatory protein 2 (IRP2) in cell culture. The stabilization of IRP2 increased the binding of IRP2 to iron response element (IREs) in transferrin receptor and ferritin mRNA, promoting transferrin receptor expression and suppressing ferritin synthesis. In support of this hypothesis, erythroleukaemia K562 cells grown in an aluminum-supplemented medium displayed an increase in surface transferrin receptors relative to controls (Perez et al. 2001). A second hypothesis is that aluminum causes cellular iron to become trapped in a subcellular compartment such as mitochondria or lysosomes, reducing the amount of cytosolic iron available to bind with IRP2 (Abreo et al. 1999). The lack of iron in the cytosol would stabilize IRP2 and affect the synthesis

of transferrin receptors and ferritin (Zatta et al. 2000, Nesse and Garbossa 2001, and Abreo and Glass 1993).

In order to better understand the effects of Al on iron metabolism, there is a need to further characterize the effects of Al on tissue iron levels and the regulation of proteins involved in cellular iron homeostasis, especially in vivo, since all work to date has been done in cell cultures. In previous studies, our laboratory observed that chicks fed aluminum had reduced ferritin levels in liver, intestine, and kidney; however, the levels of iron in these tissues and its subcellular distribution were not well characterized (Han et al. 2000). The objectives of this study were to determine, in vivo, if aluminum alters tissue iron levels and their relationship to ferritin protein expression, and the amount of iron sequestered within ferritin. Kidney iron levels, the subcellular distribution of kidney iron, kidney ferritin levels, and the amount of iron incorporated into ferritin were measured in chicks fed diets that varied in iron and aluminum content. The kidney was chosen as a model tissue in this study because it takes less time for aluminum to accumulate in the kidney than in the brain, and the regulation of intracellular iron homeostasis is similar in both tissues.

MATERIALS AND METHOD

ANIMALS, DIETS, AND EXPERIMENTAL DESIGN

Two experiments were conducted using 1 day old White Leghorn cockerels (Asagi Hatchery, Honolulu, HI). Chicks with similar body weights were randomly assigned to dietary treatment groups as describe below. The compositions of experimental diets (Table 1) were based on the reference soy isolate diet for chicks set by the National Research Council (National Research Council 1994). The chicks were housed in stainless steel wire brooders in a room with a 12-hour light: dark cycle and provided unlimited distilled water in stainless steel trays. Food intake per treatment group was recorded daily and individual body weights were measured weekly. The experimental protocol was approved by the University of Hawaii Animal Care Advisory Committee.

Experiment 1

Forty eight chicks were evenly distributed into two dietary treatment groups, control or control plus aluminum for four weeks (Table 1). The control diet contained NRC recommended amounts of iron (0.011% iron by weight) as $Fe_2^{+3}(SO_4^{-2})_3$. The control plus aluminum diet contained 0.3% aluminum in the form of $AlCl_3$-$6H_2O$ added in place of dextrose. The chicks were pair-fed their experimental diets for four weeks starting from day 1.

TABLE 1
Composition of Experiment Diets

	Diet				
	1	2	3	4	5
Ingredient	Control	Control + 0.3%Al	Intermediate Fe	Intermediate Fe + 0.3%	High Fe + 0.3%
			(g/100g)		
Soy Protein[1]	25	25	25	25	25
DL-Methionine	0.6	0.6	0.6	0.6	0.6
Glycine	0.4	0.4	0.4	0.4	0.4
Corn oil[2]	4	4	4	4	4
Dextrose	59.8	57.1	59.7	57	56.95
Cellulose	3	3	3	3	3
Choline Chloride	0.2	0.2	0.2	0.2	0.2
Vitamin mix #3[3]	1	1	1	1	1
Mineral mix #3[4]	6	6	6	6	6
$Fe_2^{+3}(SO_4^{-2})_3$	--	--	0.1	0.1	0.15
$AlCl_3$-$6H_2O$	--	2.7	--	2.7	2.7
Assayed analysis					
Total Iron	0.014	0.014	0.028	0.03	0.041
Total Aluminum	0.008	0.253	0.006	0.261	0.255

[1] Soy protein isolate (from ICN Biomedicals, Cleveland, OH) Contains 92% protein.
[2] Corn oil contains 0.02% (w/w) BHT as antioxidant.
[3] Vitamin mix follows National Research Council (NRC, 1994) formula for soy isolated diet.
[4] Mineral Mix follows National Research Council (NRC, 1994) formula for soy isolated diet.

Experiment 2

Ninety four chicks were randomly assigned to three dietary treatment groups: intermediate iron (N=30), intermediate iron plus aluminum (N=24), and high iron plus aluminum (N=24) for four weeks (Table 1). The compositions of the diets were similar to experiment 1 except for iron content. The intermediate iron diet contained 0.028% iron and the high iron diet contained 0.042% iron. $Fe_2^{+3}(SO_4^{-2})_3$ was used as the iron source. The iron and aluminum (0.3% as $AlCl_3$-$6H_2O$) were added to the diet by weight in place of dextrose. The three groups of chicks were pair-fed their experimental diets for four weeks as in experiment 1.

SAMPLING AND PROCESSING OF TISSUES

The chicks were killed by carbon dioxide inhalation after an overnight fast and blood was removed by heart puncture into additive-free vacutainers. Serum was obtained by centrifugation at 2000 x g for 10 minutes at 4°C and stored frozen (-20°C). The liver and both kidneys were excised. Upon removal the livers and samples of the right lobe (0.5-1.0g) were weighed, frozen, and stored at -20°C. Kidneys were kept on ice until processed as described below.

Kidneys from individual chicks were pooled (N= 3 chicks per pooled sample) within treatment groups to yield about 6g of tissue per sample. The pooled samples were fractionated by differential centrifugation into a nuclei/cell debris fraction, mitochondrial fraction, and post-mitochondrial supernatant following established procedures (Lash and Sall 1993) as described next. The samples were homogenized (Dounce Homogenizer, Fisher Science) in five volumes of isolation buffer (20 mM Tris-HCl, pH 7.4, 1 mM

phenylmethysulfonyl fluoride, 3 mM potassium phosphate, 225 mM sucrose, 5 mM magnesium chloride, 20 mM potassium chloride) using seven up and down motions. Aliquots of the homogenate were taken for protein and iron assays. The homogenate was centrifuged at 600 x g for 10 minutes at 4°C (Avanti® J-30I, Beckman Instruments). The supernatant (1a) and pellet fractions (debris pellet) were separated. The debris pellets were resuspended in 10 ml of isolation buffer, vortexed, and recentrifuged at 600 x g for 10 minutes. The resulting supernatant was combined with supernatant 1a. The washed debris pellets were mixed with 1 volume of isolation buffer, vortexed, and an aliquot was taken for protein and iron assays. Supernatant 1a was fractionated further by centrifugation at 15,000 x g for five minutes at 4°C. The resulting supernatant (1b) and pellet fractions (mitochondria pellet) were separated. The mitochondrial pellets were resuspended in 10 ml of isolation buffer, vortexed, and recentrifuged at 15,000 x g for five minutes. The washed mitochondrial pellets were mixed with 1 volume of isolation buffer, vortexed, and an aliquot taken for protein and iron assays. The supernatant was combined with supernatant 1b and an aliquot was taken for protein and iron assays. The remainder of this post-mitochondrial supernatant was stored at -80°C for subsequent ferritin assays.

IRON AND PROTEIN ASSAYS

Liver samples (0.5-1 g), aliquots of serum (3-5 ml), kidney homogenate (3g), nuclear pellets (10-12 g), mitochondrial pellets (2-4 g), and post-mitochondrial supernatant (15 ml) were analyzed for iron content by first drying the samples overnight in a vacuum oven. The next day, the samples were wet-ashed in 2 ml of 50% trace metal

grade nitric acid on a hot plate and diluted to 10 ml with 1% HCl. The samples were sent to the University of Hawaii at Manoa Agricultural Diagnostic Service Center which measured iron by ICP emission spectroscopy. Iron content in the liver and kidney was expressed as μg Fe/g tissue and in the subcellular fractions as μg Fe/g pellet weight or μg Fe/g supernatant. A bovine liver standard reference sample (National Institute of Standards and Technology, SRM 1577b) prepared the same way as the samples was assayed for iron to validate procedures. A recovery of 103±5% (n=6) was obtained. Iron and aluminum contents of experimental diets were also measured by ICP emission spectroscopy after dry-ashing overnight at 500°C followed by wet ashing, as above, then diluted to 50 ml with 1% HCl.

The protein content of all fractions was quantified by absorption spectrophotometry (Shimadzu UV-Visible Recording Spectrophotometer, UV-160) at 750 nm using the Modified Lowry Protein Assay (Pierce Biotechnology, Inc., Rockford, IL).

FERRITIN PROTEIN AND IRON CONTENT BY NATIVE-PAGE

The post-mitochondrial supernatant was thawed, heat-treated at 100°C for five minutes, cooled in ice, centrifuged for 30 minutes at 15,000 x g, and the supernatant concentrated by centrifugal ultrafiltration at 4°C (Centricon 10, Millipore Corporation). Protein analyses on the concentrated samples were performed using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

The relative amounts of ferritin protein in the kidney supernatants and the ratio of ferritin protein to ferritin bound iron were determined from ferritin bands resolved by

native-PAGE. Aliquots of concentrated supernatant samples were diluted in equal volumes of native-PAGE sample buffer (Bio-Rad Laboratories) and loaded onto a discontinuous polyacrylamide gel (10% acrylamide separating and 4% acrylamide stacking gel). Equal amounts of supernatant protein from each treatment group were loaded onto the gels. The proteins were fractionated using a Mini-Protean® 3 vertical slab gel (Bio-Rad Laboratories) performed at 15 mAmps per gel for 3.5 hours according to the manufacture's instructions.

Identical gels were run to assay ferritin protein and ferritin bound iron. To determine relative amounts of ferritin protein, protein bands in the gels were stained with coomassie (0.0025% coomassie blue R-250, 40% methanol, 7% acetic acid) for 2 hours and the gel was destained (40% methanol, 7% acetic acid for 1 hour and 7% acetic acid, 5% methanol until clear). Images of gels were taken after destaining using a Flour-S™ Multilmager (Bio-Rad Laboratories) and band intensities were analyzed by Bio-Rad Quantity One Quantitation Software Version 4.2 (Bio-Rad Laboratories). The location of the ferritin protein band on the gel was verified by comparison to 5μg of a horse spleen ferritin standard (Sigma, St. Louis, MO). Ferritin band intensities were normalized across different gels by expressing band intensity as a ratio to an internal reference sample chosen from the intermediate iron group. This reference standard was run on all gels.

Iron containing protein bands in the gel were visualized using an iron staining solution consisting of a mixture of 0.75 mM Ferene S (Sigma, St. Louis, MO) and 15 mM thioglycolic acid in 2% (v/v) acetic acid (Chung 1985). For color development, each gel was immersed in 50 ml of staining solution for 30 minutes. The gel background could be

cleared by destaining in 2% (v/v) acetic acid. Images of gels were taken after 15 minutes of destaining and band intensities were analyzed using a Flour-S™ Multilmager (Bio-Rad Laboratories). Locations of ferritin bands on the gel were verified by comparison to 2 μg of a horse spleen ferritin standard (Sigma, St. Louis, MO). The intensity of iron staining was normalized across gels by expressing it as a ratio to the same internal reference standard used above for protein.

STATISTICAL ANALYSIS

All analyses were conducted using SAS version 8 software (SAS Institute, Cary, NC). Results were expressed as means ±SD. The effects of dietary treatments on all measured parameters were analyzed by one-way ANOVA and when the ANOVA indicated a significant effect ($p<0.05$), the Tukey's Studentized Range (HSD) test was used to compare treatment means. Because dietary treatments were found to significantly affect kidney iron levels and kidney iron is known to affect ferritin synthesis, ferritin bound iron and the amount of iron in subcellular fractions, we further analyzed these data using kidney iron as a covariable in an analysis of covariance. This allowed the effects of aluminum to be analyzed independent of any effects due to kidney iron. Linear regression analyses of the relationship between kidney iron and serum iron, ferritin protein, ferritin bound iron, and iron in subcellular fractions were conducted using the combined data from experiments I and II. One regression line was fit to data from the Al-treated groups, another line was fit to the data from the non-Al treated groups. The two regression lines (with aluminum treatment and without aluminum treatment) were

compared for treatment effects and differences in slope using analysis of covariance. A value of $p<0.05$ was considered significant in all analyses.

RESULTS

FOOD INTAKE AND BODY WEIGHT GAIN

Table 2 summarizes the data on food intake and body weight gain. Since aluminum is known to reduce food intake in the chick (Dunn et al. 1993), pair-feeding was conducted within each experiment to prevent large differences in food intake and iron intake. Food intake was about 18% lower in experiment II than in experiment I, apparently due to the combined effects of supplemental iron and aluminum. Supplemental iron alone was found to reduce food intake, but the group consuming high Fe plus 0.3% Al ate the smallest amount of food and was the group pair-feeding was based on. Weight gain was about 37% lower in experiment II (250g vs. 158g) than experiment I reflecting the reduced food intake and lower gain/food intake ratio of all groups in experiment II. Within each experiment, aluminum did not have a significant effect on body weight gain.

WEIGHT OF LIVER, KIDNEYS, AND KIDNEY SUBCELLULAR FRACTIONS

Kidney and Liver

Table 3 shows the effect of dietary iron and aluminum on the weights of liver, kidneys, and kidney subcellular fractions. No changes were detected in kidney weight due to different levels of iron, but the weight increased with aluminum in the diet at the control and intermediate levels of iron. Kidney weight in the high iron + Al group,

TABLE 2

Body Weight Gain and Food Intake of Chicks Fed Various Levels of Fe and Al in Diets

Treatment	N	Weight Gain (g/bird)[1]	Food Intake (g/bird)[2]	Iron Intake (mg/bird)[3]	Food Efficiency ratio (g gain:g food intake)
Experiment I					
Control	24	257±42[a]	403	55	0.64
Control + 0.3% Al	24	244±37[a]	403	55	0.61
Experiment II					
Intermediate Fe	36	160±21[b]	320	91	0.50
Intermediate Fe+0.3% Al	36	158±25[b]	340	102	0.46
High Fe + 0.3%Al	24	156±27[b]	322	130	0.48

[1] Values are means ± SD. Means in a column without a common superscript letter (a, b) are statistically different ($p<0.05$) by Tukey's Studentized (HSD) Test.

[2] Within each experiment, treatment groups were pair-fed. Values are the mean intake per bird over the 4 week period.

[3] Values are mean intake per bird for the total 4 week period.

TABLE 3

Effects of Dietary Iron and Aluminum Intake on Kidney Weight, Liver Weight, and the Weight and Protein Concentration of Kidney Subcellular Fractions[1]

					Kidney Subcellular Fractions					
	Kidney Weight		Liver Weight		Debris Pellet		Mitochondrial Pellet		Supernatant	
Diet	Weight[2]	% Body Weight	Weight	% Body Weight	Weight	Protein	Weight	Protein	Weight	Protein
	(g)	(%)	(g)	(%)	(%)[3]	(mg/g)[4]	(%)[3]	(mg/g)[4]	(%)[3]	(mg/g)[4]
Control	2.0±0.2^{a}	0.7±0.1^{a}	6.5±0.7^{a}	2.3±0.2^{a}	11.8±1.7^{ab}	36.9±1.2^{a}	3.9±0.9^{a}	60.3±7.4^{a}	84.3±1.3^{ab}	9.7±1.2^{a}
Control + Al	2.8±0.4^{b}	1.0±0.1^{b}	6.4±1.0^{a}	2.3±0.1^{ab}	12.0±1.4^{b}	37.3±1.8^{ac}	4.1±0.3^{a}	62.3±5.4^{a}	83.9±1.2^{b}	10.9±0.8^{a}
Intermediate Fe	1.9±0.1^{a}	1.0±0.1^{b}	5.0±0.1^{b}	2.5±0.1^{bd}	11.5±0.6^{ab}	41.3±1.2^{b}	3.6±0.5^{a}	64.4±4.7^{a}	84.8±0.3^{ab}	9.8±0.8^{a}
Intermediate Fe + Al	3.2±0.4^{c}	1.6±0.1^{c}	6.5±0.9^{a}	3.3±0.2^{c}	9.5±0.3^{c}	--	5.5±0.7^{b}	--	85.0±0.6^{ab}	--
High Iron + Al	1.9±0.2^{a}	1.0±0.1^{b}	5.0±0.5^{b}	2.6±0.2^{d}	10.4±0.3^{ac}	39.5±1.7^{bc}	4.2±0.4^{a}	65.2±2.8^{a}	85.4±0.3^{a}	9.7±0.7^{a}

[1]Values are means ±SD (n=7 for Control and Control+Al, n=8 for High Fe+Al, n=12 for Intermediate Fe and Intermediate Fe+Al). Means in a column without a common superscript letter are significantly different ($p<0.05$).

[2] Weight of both kidneys (pooled from 3 chicks divided by 3)

[3] % of kidney homogenate weight

[4] mg protein/g wet weight of fraction

however, was not greater than the control group. This suggests that a high level of iron in the diet counteracted the effect of aluminum on increasing kidney weight. Similar results were found when kidney weight was expressed as % of body weight.

The weight of the liver was significantly reduced as the level of dietary iron increased, possibly due to the smaller weight gain in experiment II since liver weight expressed as % body weight was minimally effected by iron intake. Aluminum had no effect on liver weight at the control level of iron intake, but increased liver weight at the intermediate level. In the high iron + Al group the livers were again smaller. This implied, as in the kidney, that aluminum may increase liver weight and that a high level of iron counteracts the effects of aluminum.

Subcellular Fractions

Overall, neither increased iron intake nor aluminum intake greatly altered the subcellular composition of the kidney (Table 3). Therefore, even though aluminum increased kidney weight, it did not alter the composition of the kidney, nor did it alter the protein content of the subcellular fractions.

IRON CONTENT OF SERUM, LIVER, KIDNEYS, AND KIDNEY SUBCELLULAR FRACTIONS

Serum, Liver, and Kidneys

There was an increase in the concentration of iron in the liver and kidneys as the iron concentration in the diet increased from control to intermediate levels (Table 4). Adding aluminum to these diets, however, greatly reduced iron concentrations and

TABLE 4

Effects of Dietary Iron and Aluminum Intake on Liver, Kidney, and Serum Iron Concentration and the Distribution of Iron in Kidney Subcellular Fractions[1]

Diet	Kidney Iron	Liver Iron	Serum Iron	Iron in Kidney Subcellular Fractions		
				Debris Pellet	Mitochondrial Pellet	Supernatant
	(μg Fe/g)[2]	(μg Fe/g)[2]	(μg Fe/ml)	(μg Fe/g)[3]	(μg Fe/g)[3]	(μg Fe/g)[4]
Control	48±4^{a}	63±8^{a}	1.6±0.2^{a}	20±3^{a}	21±4^{ac}	1.9±0.3^{a}
Control + Al	38±7^{b}	54±7^{a}	1.0±0.3^{b}	16±5^{a}	16±4^{b}	1.6±0.2^{a}
Intermediate Fe	58±6^{c}	79±7^{b}	1.7±0.4^{a}	26±3^{b}	21±3^{a}	2.3±0.3^{c}
Intermediate Fe + Al	34±4^{b}	51±15^{a}	1.1±0.3^{bc}	18±3^{a}	17±2^{bc}	1.1±0.2^{b}
High Fe + Al	51±6^{ac}	–	1.5±0.2^{ac}	30±3^{b}	20±2^{ab}	2.0±0.3^{a}

[1]Values are means ±SD (n=7 for Control and Control+Al, n=8 for High Fe+Al, n=12 for Intermediate Fe and Intermediate Fe+Al). Means in a column without a common superscript letter are significantly different ($p<0.05$).

[2] μg Fe/g wet weight

[3] μg Fe/g wet pellet

[4] μg Fe/g supernatant

prevented the increase in tissue iron except in the high iron + Al group. Aluminum also reduced serum iron concentrations, except in the high iron + Al group. This suggests that reduced tissue iron may be related to reduced serum iron, and that high dietary iron counteracts the ability of aluminum to reduce serum and tissue iron levels.

The effect of aluminum on the relationship between serum iron and kidney iron was investigated using analysis of covariance on the combined data from experiments I and II (Figure 1). Serum iron was used as the covariable. The results showed that kidney iron was significantly related to serum iron only in the aluminum treated group ($p=0.0001$) with the slope being significantly larger than the iron group (slope $p=0.03$). However, when just the data representing normal serum iron levels (above 1.25μg/ml) were compared (Figure 1, inset), there was no longer a difference in slope due to aluminum, and no significant treatment effect due to aluminum was found. Therefore, the regression lines were not different over this range of serum iron. This indicates that above 1.25μg Fe/ml serum, aluminum was not altering the relationship between serum iron and kidney iron content. If aluminum is not directly affecting the uptake of serum iron into the kidney, it suggests that decreases in kidney iron concentrations may be the result of low serum iron levels possibly brought about by aluminum inhibiting intestinal iron absorption. When dietary iron is high enough, it overcomes the inhibitory effect of aluminum on iron absorption, and normal serum iron concentration and tissue levels are obtained.

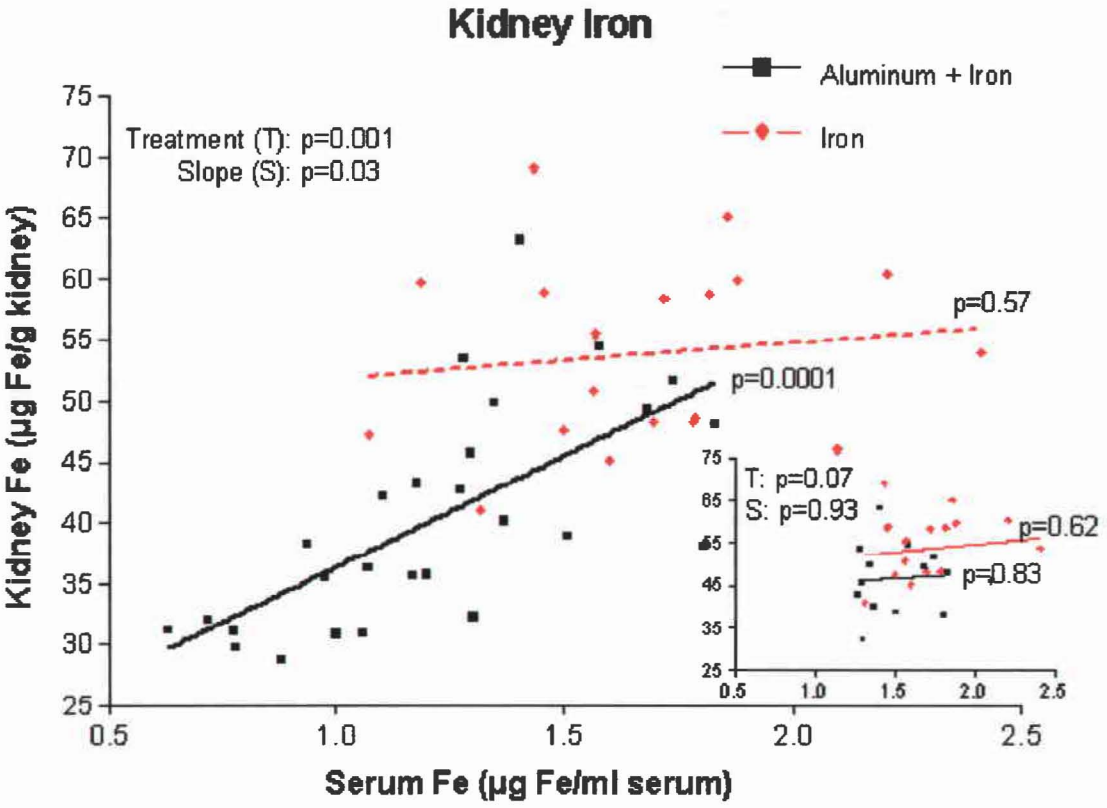


FIGURE 1 Effects of dietary treatments on the relationship between serum iron and kidney iron. Chicks were fed diets containing different levels of iron with added aluminum (■) or without added aluminum (♦). Kidney and serum iron concentrations were measured. The effects of dietary treatments on the relationship between serum iron and kidney iron was investigated using analysis of covariance using the combined data from both experiments. A p-value ≤0.05 for a regression line indicates that the slope of the line is significantly different from zero. A p-value ≤0.05 for slope (S) indicates that the slopes of the two regression lines are significantly different. A p-value ≤0.05 for treatment (T) indicates that the regression lines are significantly different from each other. Inset: The relationship between serum iron and kidney iron in the treatment groups when serum iron was greater than 1.25 µg Fe/ml serum.

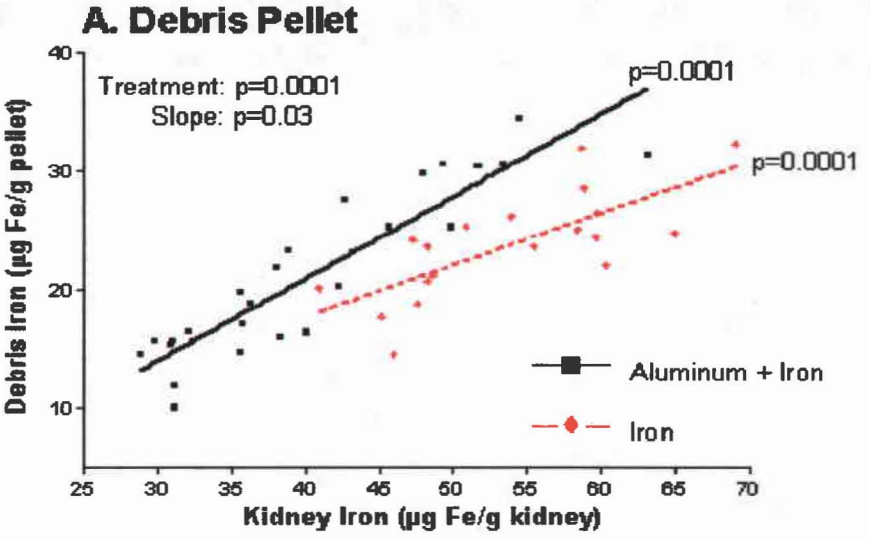
Subcellular Fractions

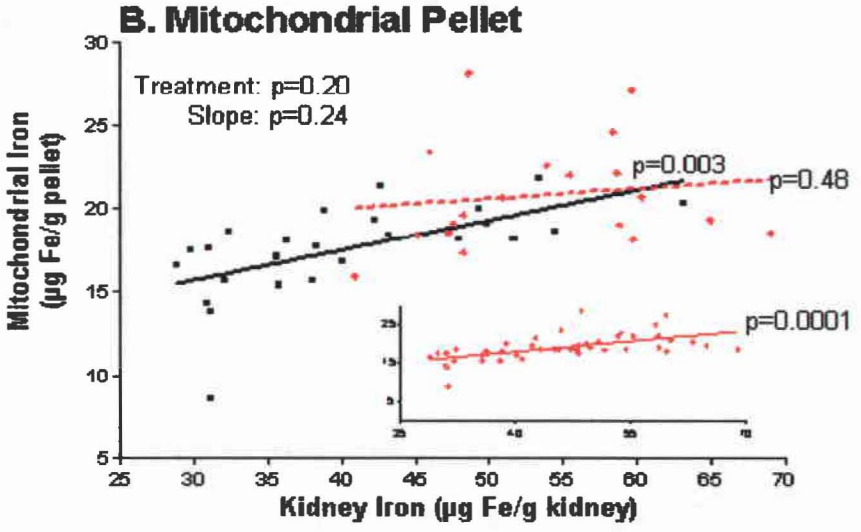
Debris Pellet. The level of iron in the debris pellet increased as the level of iron in the diet and the concentration of iron in the kidney increased (Table 4). This indicated that debris pellet iron concentrations were related to kidney iron content. Therefore, to analyze the effects of aluminum on debris pellet iron concentrations independent of aluminum's effect on kidney iron, analysis of covariance was used. Debris pellet iron was plotted (as a dependent variable) against kidney iron content, and regression lines fitted to the data from aluminum-treated and non-treated (iron) groups were compared (Figure 2A).

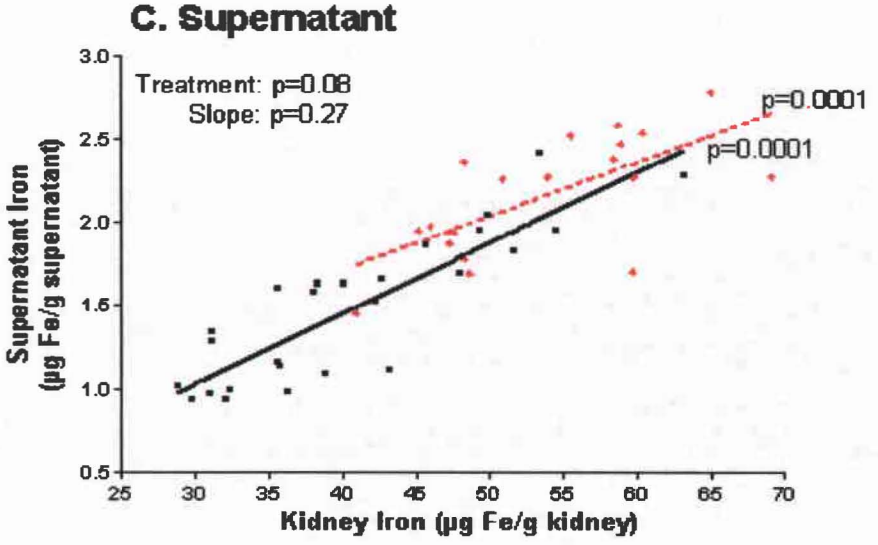
The results indicated that debris pellet iron was significantly correlated with kidney iron in both the aluminum treated ($p<0.0001$) and non-treated groups ($p<0.0001$). The slope of the regression line was greater, however, for the Al-treated group (slope $p=0.028$). This indicates that aluminum increased the rate of accumulation of iron in the debris pellet resulting in higher concentrations of iron in this fraction, especially when kidney iron content was in the higher range (e.g. above 50 μg/g).

Mitochondrial pellet. Increasing the iron level in the diet did not affect the concentration of iron in the mitochondrial pellet (Table 4). The mean iron concentration in the mitochondrial fraction was reduced, however, by adding aluminum to the control and intermediate iron diets, but this may have been related to the low concentration of kidney iron in these groups (Table 4). When analyzed by analysis of covariance (Figure 2B) no difference was found between groups with and without aluminum in the relationship of mitochondrial iron to kidney iron (slope $p=0.24$, treatment $p=0.199$).

FIGURE 2 Effects of dietary treatments on the relationship between kidney iron and the concentration of iron in the debris pellet, mitochondrial pellet, and supernatant. Chicks were fed diets containing different levels of iron with or without added aluminum. The debris pellet, mitochondrial pellet, and supernatant fractions were isolated from the kidney and iron levels were measured in each fraction. The relationship between kidney iron and the iron in each subcellular fraction was investigated using analysis of covariance on the combined data from both experiments. (A) Effects of dietary treatments [(iron (♦) or iron + Al (■))] on the relationship between kidney iron and debris pellet iron. (B) Effects of dietary treatments on the relationship between kidney iron and mitochondrial pellet iron. Inset: the regression line fitted to the combined data from both the iron and iron + Al groups. (C) Effects of dietary treatments on the relationship between kidney iron and supernatant iron concentration. A p-value ≤ 0.05 for the regression line, slope (S) and treatment (T) indicate respectively: that the slope of the line is different from zero, that the slope of the two lines are significantly different from each other, and that the two regression lines are significantly different.


A. Debris Pellet
Treatment: p=0.0001
Slope: p=0.03
p=0.0001
p=0.0001
Aluminum + Iron
Iron
Debris Iron (µg Fe/g pellet)
Kidney Iron (µg Fe/g kidney)


B. Mitochondrial Pellet
Treatment: p=0.20
Slope: p=0.24
p=0.003
p=0.48
p=0.0001
Mitochondrial Iron (µg Fe/g pellet)
Kidney Iron (µg Fe/g kidney)


C. Supernatant
Treatment: p=0.08
Slope: p=0.27
p=0.0001
p=0.0001
Supernatant Iron (µg Fe/g supernatant)
Kidney Iron (µg Fe/g kidney)

Therefore, a regression line was fit to the combined data from all groups (Figure 2B inset). The resulting slope indicated that mitochondrial iron had a small positive relationship to kidney iron ($p=0.0001$) suggesting that the effects on mitochondrial iron seen in Table 4 are not due to Al, but due to the changes in kidney iron concentrations.

Supernatant. The concentration of iron in the supernatant increased with the level of iron in the diet and the level of iron in the kidney (Table 4). Adding aluminum to the diets appeared to reduce supernatant iron, but this was found by analysis of covariance to be related to the reduced kidney iron content in the aluminum groups (Figure 2C). Supernatant iron was positively related to kidney iron in both the non-treated and Al-treated groups ($p=0.0001$), but aluminum had no significant effect on this relationship (slope $p=0.27$, treatment $p=0.08$). This suggests that changes in supernatant iron are due to changes in kidney iron and not direct effects of aluminum.

KIDNEY FERRITIN AND FERRITIN BOUND IRON

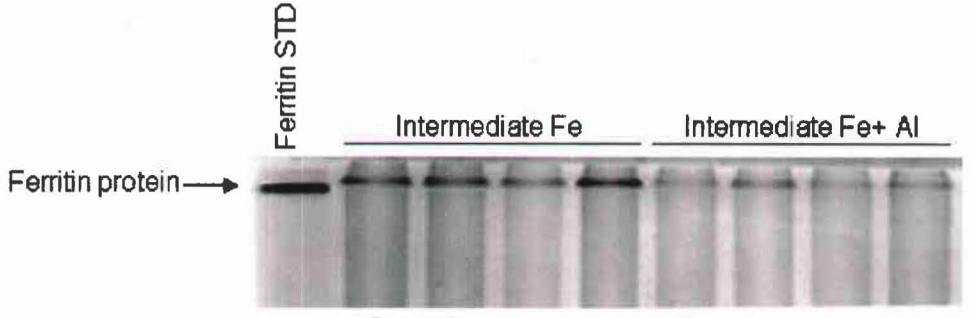
Representative native PAGE gels illustrating the assay for the relative amounts of ferritin bound iron and ferritin protein in kidney supernatant fractions are shown in Figures 3A and 3B, respectively. Shown are band intensities for 4 samples from the intermediate iron group (samples1-4) and 4 samples from the intermediate Fe + Al group (samples 5-8). The same samples were run on gels A and B. Band intensities on both gels were expressed as a ratio to the band intensity of sample #1 (the reference sample for all gels). Also shown in Figure 3B is the ratio of ferritin bound iron to ferritin protein expressed as the band intensity for ferritin iron divided by the band intensity for ferritin

FIGURE 3 Representative native PAGE gels showing the assays for relative amounts of ferritin iron and ferritin protein in kidney supernatants.
(A) Representative gel assay for ferritin bound iron. Heat stable supernatant proteins (100 μg) from chicks fed the intermediate Fe diet (samples 1-4) and intermediate Fe + Al diet (samples 5-8) were separated by native-PAGE and stained with an iron staining solution. Band intensities were express as a ratio to the band intensity of sample #1 (the reference sample for all gels). A horse spleen ferritin standard (2 μg) is shown in the last lane. (B) Representative gel assay for ferritin protein. Heat stable supernatant proteins (10 μg) from the same samples as gel A were separated by native-PAGE and stained with coomassie. Band intensities were expressed as a ratio to sample #1 (the reference sample for all gels). A horse spleen ferritin standard (5 μg) is shown in the first lane. The ratio of ferritin iron to ferritin protein expressed as the band intensity for ferritin iron divided by the band intensity for ferritin protein is also shown.

A

Sample #:	1	2	3	4	5	6	7	8
Kidney Fe (µg/g kidney):	65	58	60	61	39	36	29	30
Fe band intensity (ratio to ref. STD):	1	1.08	1.15	1.32	0.37	0.39	0.26	0.15

B



Sample #:	2	3	1	4	5	6	7	8
Protein band intensity (ratio to ref. STD):	1.24	1.17	1	1.19	0.64	0.74	0.63	0.62
Ratio of ferritin Fe to ferritin protein	0.87	0.98	1	1.11	0.58	0.53	0.41	0.24

protein. These measurements were made on gels from all treatment groups and the results are summarized in Table 5, and Figures 4-6 (individual gels are shown in Appendix A).

The intermediate iron diet was found to increase kidney ferritin-bound iron 200% relative to controls, while ferritin protein increased only 64% (Table 5). This resulted in a 100% increase in the ratio of ferritin-bound iron to ferritin protein due to increased levels of iron in the kidney. Aluminum appeared to reduce ferritin protein, ferritin-bound iron, and ferritin iron to protein ratios in Table 5, especially at the intermediate iron level. However, since kidney iron affects ferritin protein and ferritin iron levels, the effects of aluminum on these parameters were investigated further by analysis of covariance using kidney iron as the covariable (Figures 4-6).

Aluminum did not have an effect on the rate of increase in ferritin protein as kidney iron increased (Figure 4). However, aluminum did affect the amount of iron incorporated into ferritin (Figure 5). As kidney iron increased, the rate of increase in ferritin iron was significantly less in the Al-treated group compared to the group without Al (slope $p=0.001$, Figure 5A). When only the data from animals with kidney iron above 40μg/g were used in the analysis (to more directly compare the response over the range where kidney iron levels were the same between treatments) the slopes became the same, i.e., were parallel (Figure 5A, inset). However, there was a treatment effect indicating that the regression lines were different and that, at the same concentration of kidney iron, less iron was taken up into ferritin in the aluminum group. This inhibiting effect of aluminum on ferritin iron accumulation was also apparent when supernatant iron concentrations were used as the covariable instead of kidney iron concentrations (Figure

TABLE 5

Effects of Dietary Iron and Aluminum Intake on Ferritin Bound Iron, Ferritin Protein, and the Ratio of Ferritin Bound Iron: Ferritin Protein in Chick Kidney[1]

Diet	Relative Ferritin Bound Iron Levels[2]	Relative Ferritin Protein Levels[3]	Ferritin Iron: Ferritin Protein[4]
Control	0.31 ± 0.08^{ab}	0.64 ± 0.16^{a}	0.46 ± 0.14^{bc}
Control + Al	0.15 ± 0.05^{b}	0.52 ± 0.23^{a}	0.28 ± 0.1^{c}
Intermediate Fe	0.95 ± 0.24^{c}	1.05 ± 0.08^{b}	0.93 ± 0.24^{a}
Intermediate Fe + Al	0.43 ± 0.14^{a}	0.66 ± 0.19^{a}	0.61 ± 0.27^{b}
High Fe + Al	0.43 ± 0.15^{a}	0.97 ± 0.1^{b}	0.42 ± 0.17^{bc}

[1] Values are means ±SD (n=7 for Control and Control+Al, n=8 for High Fe+Al, n=12 for Intermediate Fe and Intermediate Fe+Al). Means in a column without a common superscript letter are different ($p \leq 0.05$).

[2] Ratio of sample band intensity to reference sample band intensity (reference sample was from the Intermediate Fe group).

[3] Ratio of sample band intensity to reference sample band intensity (reference sample was from the Intermediate Fe group).

[4] The band intensity ratio for ferritin iron divided by the band intensity ratio for ferritin protein.

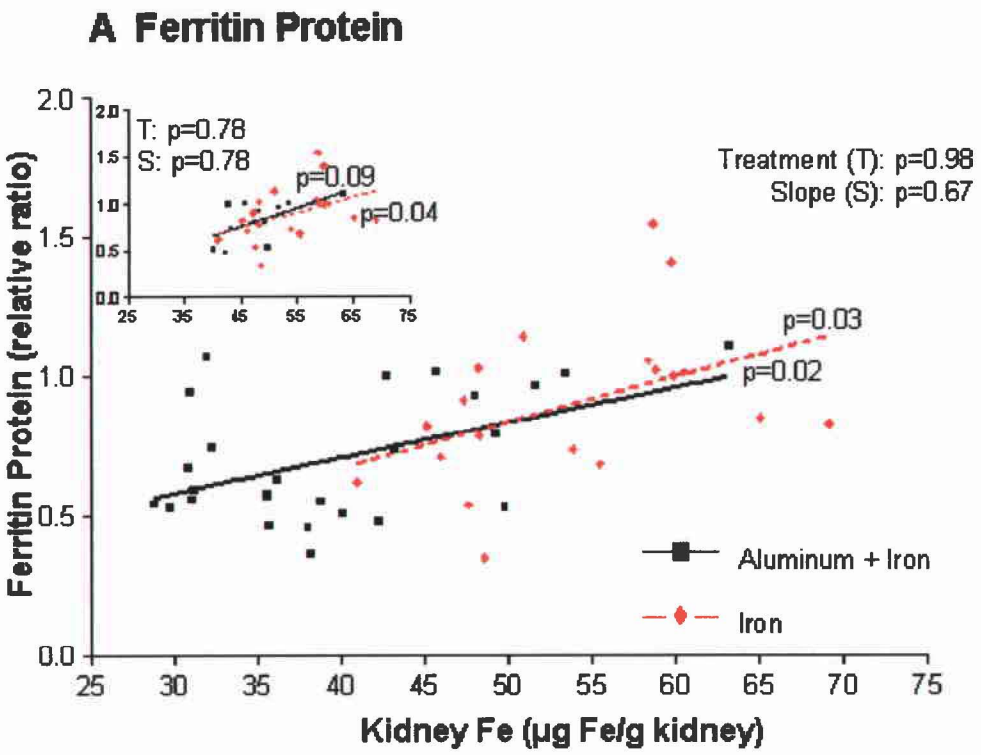
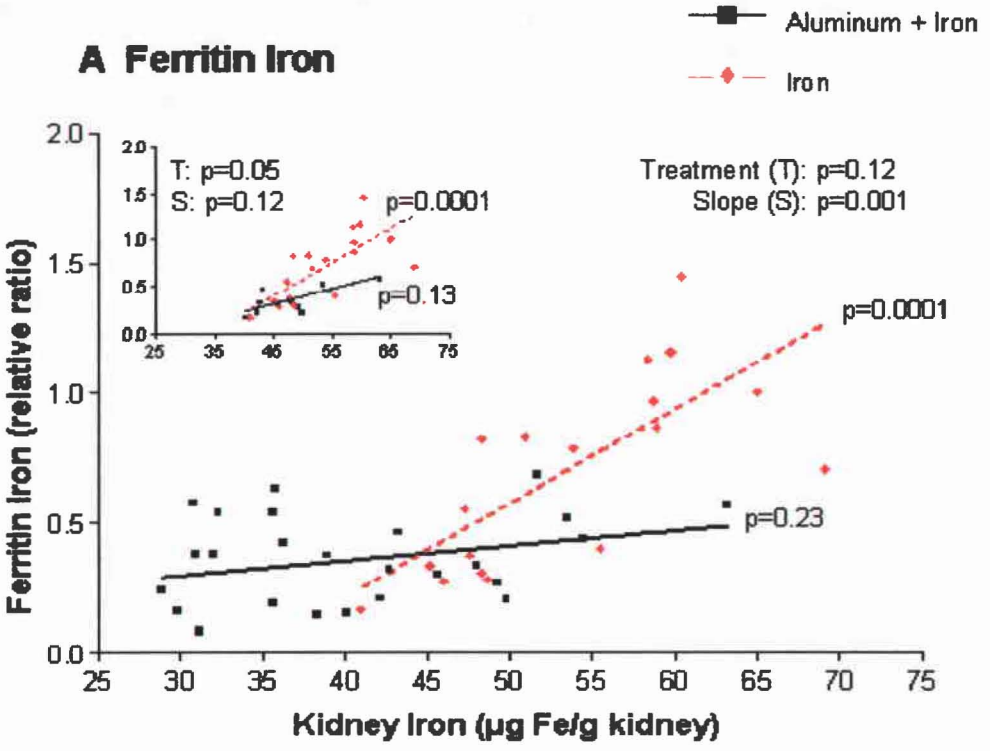


FIGURE 4 Effects of dietary treatments on the relationship between kidney iron and ferritin protein. Chicks were fed diets containing different levels of iron with or without added aluminum. Ferritin protein levels in kidney supernatant fractions were determined by native PAGE and plotted against kidney iron levels. The effects of dietary treatments on the relationship between kidney iron content and ferritin protein levels were analyzed by analysis of covariance using the combined data from both experiments. A p-value ≤ 0.05 for the regression line, slope (S) and treatment (T), indicate respectively: that the slope of the line is significantly different from zero, that the slopes of the two lines are significantly different from each other, and that the two regression lines are significantly different.

FIGURE 5 Effects of dietary treatments on the relationship between kidney iron and ferritin bound iron. Chicks were fed diets containing different levels of iron with or without added aluminum. Ferritin bound iron concentrations were measured in the kidney supernatant fractions by native PAGE and plotted against kidney iron levels. (A) Effects of dietary treatments on the relationship between kidney iron and ferritin bound iron analyzed by analysis of covariance using the combined data from both experiments. Inset: the relationship between kidney iron and ferritin iron when kidney iron was above 40ug Fe/g kidney. (B) Effects of dietary treatments on the relationship between kidney supernatant iron concentration and ferritin bound iron. Inset: the relationship between supernatant iron and ferritin iron when supernatant iron was above 1.4 μg Fe/g supernatant. A p-value ≤ 0.05 for the regression line, slope (S) and treatment (T) indicate respectively: that the slope of the line is different from zero, that the slope of the two lines are significantly different from each other, and that the two regression lines are significantly different.


A Ferritin Iron
Aluminum + Iron
Iron
Treatment (T): p=0.12
Slope (S): p=0.001
T: p=0.05
S: p=0.12
p=0.0001
p=0.13
p=0.0001
p=0.23
Ferritin Iron (relative ratio)
Kidney Iron (µg Fe/g kidney)

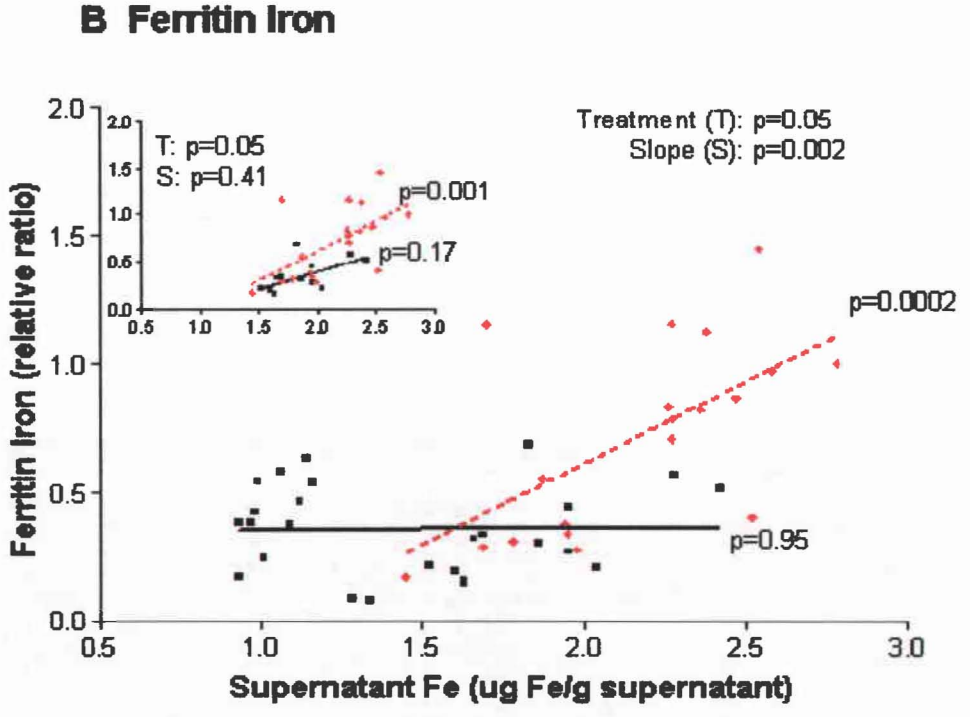
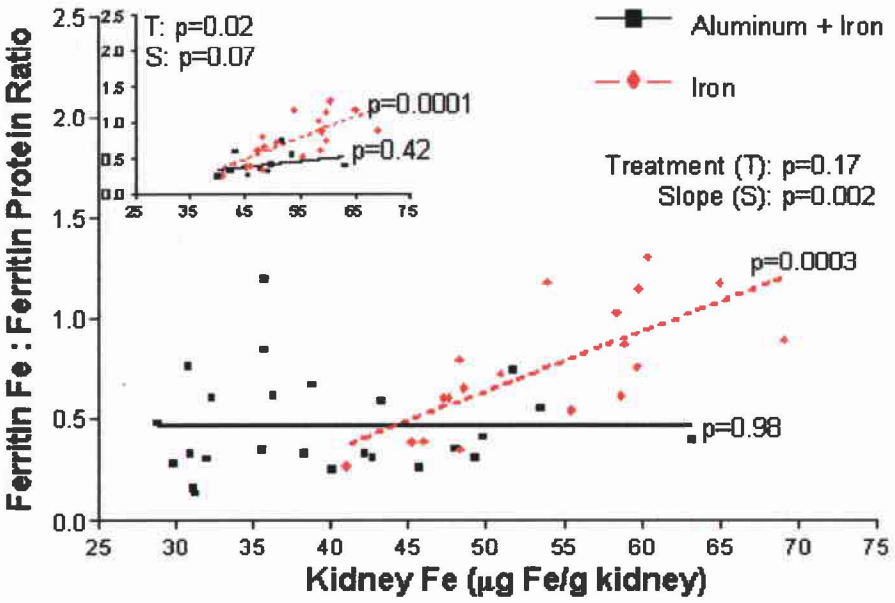
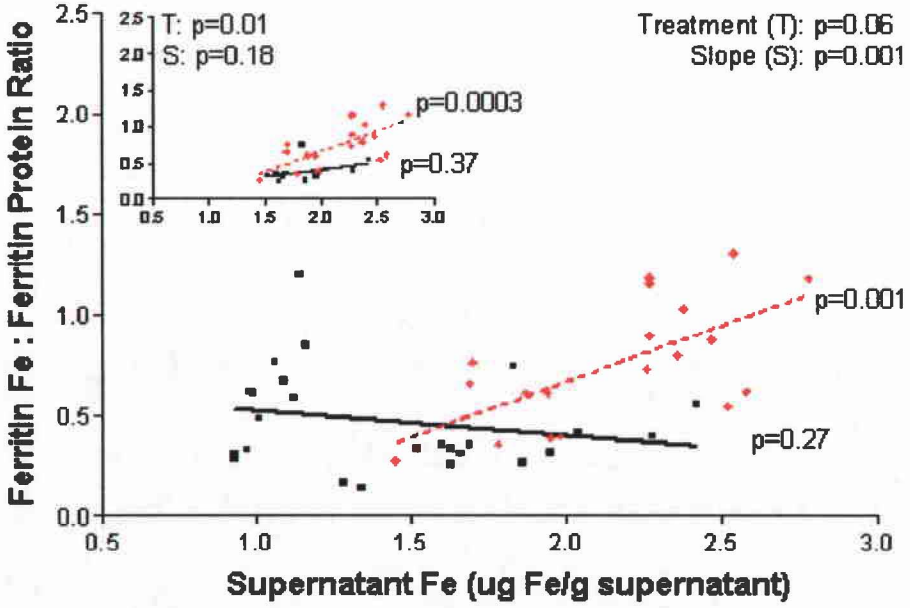

B Ferritin Iron
Treatment (T): p=0.05
Slope (S): p=0.002
T: p=0.05
S: p=0.41
p=0.001
p=0.17
p=0.0002
p=0.95
Ferritin Iron (relative ratio)
Supernatant Fe (ug Fe/g supernatant)

FIGURE 6 Effects of dietary treatments on the relationship between kidney iron and the ratio of ferritin iron to ferritin protein. Chicks were fed diets containing different levels of iron with or without added aluminum. The ratio of ferritin bound iron to ferritin protein in kidney supernatant fractions was determined by native PAGE and plotted against kidney iron levels. (A) Effects of dietary treatments on the relationship between kidney iron and the ratio of ferritin iron : ferritin protein analyzed by analysis of covariance using the combined data from both experiments. Inset: the relationship between kidney iron and the ratio of ferritin iron : ferritin protein when kidney iron was above 40 μg Fe/g kidney. (B) Effects of dietary treatments on the relationship between kidney supernatant iron and the ratio of ferritin iron : ferritin protein. Inset: the relationship between supernatant iron and the ratio of ferritin iron : ferritin protein when the kidney supernatant iron concentration was above 1.4 μg Fe/g supernatant. A p-value ≤ 0.05 for the regression line, slope (S), and treatment (T) indicate respectively: that the slope of the line is significantly different from zero, that the slopes of the two lines are significantly different from each other, and that the two regression lines are significantly different.

A Ratio of Ferritin Iron to Ferritin Protein



B Ratio of Ferritin Iron to Ferritin Protein



5B). Supernatant iron was used since it is presumably the source of ferrous ions for uptake into cytosolic ferritin.

Since aluminum reduced iron uptake into ferritin, we also investigated the effect of aluminum on the ratio of ferritin iron to ferritin protein (Figure 6). In the groups without aluminum, the iron to protein ratio increased as kidney iron increased. Aluminum, however, was found to reduce the iron to protein ratio in ferritin. This result is analogous to aluminum's effect on iron uptake into ferritin and was also present when supernatant iron was used as the covariable (Figure 6B).

DISCUSSION

This study demonstrated that dietary aluminum toxicity can negatively impact iron metabolism in ways that could impair cell function, and that the effects differ somewhat from those seen in cell culture studies. Dietary aluminum reduced iron concentrations in tissues (serum, liver, and kidneys). When effects due to reduced tissue iron were corrected for, aluminum was found to inhibit iron uptake into ferritin, but did not alter total iron concentrations in the cytosol or ferritin protein levels. In contrast, cell culture studies indicate that aluminum increases cellular iron concentrations, reduces both ferritin protein levels and ferritin iron uptake, and causes iron to be partitioned out of the cytosol accumulating in mitochondria and nuclei (Abreo et al. 1994, 1999, and Yamanaka et al. 1999). These contrasting findings suggest that cell culture studies may not fully reflect the effects of dietary aluminum toxicity in vivo. The possible reasons behind these contrasting findings will be discussed below, as well as the potential

mechanisms for the effects of aluminum on iron metabolism in vivo and their significance.

Our results indicate that high intakes of aluminum most likely decrease tissue iron levels because aluminum inhibits non-heme iron absorption by the intestine. Non-heme iron (ferric sulfate) was the only source of dietary iron used in our study. A reduction in non-heme iron absorption would reduce serum iron levels and, therefore, delivery of iron to tissues. Cell culture studies would not be exposed to this effect. Apparently, reduced delivery of iron to tissues outweighed any stimulatory effect aluminum may have had on transferrin receptor expression and tissue iron uptake shown to occur in tissues based on cell cultures studies (Abreo et al. 1999 and Yamanaka et al. 1999).

We did not measure intestinal iron absorption directly, so our conclusion that dietary aluminum inhibits non-heme iron absorption is based on the fact that there are few apparent reasons, other than reduced iron absorption, for serum iron to be low. Other causes of reduced serum iron are excessive iron loss from the body, an expanded plasma volume, and increased uptake of serum iron into tissues or erythrocytes. Our data do not support increased tissue uptake of iron since liver and kidney iron concentrations were reduced by aluminum and previous studies in the chick suggest that intestinal iron levels are also reduced by dietary aluminum (Han et al. 2000). With respect to iron loss from the body, the only significant route of iron loss is blood loss, which was not seen in this study. In regard to increased plasma volume, aluminum toxicity is known to cause microcytic anemia and reduced hematocrits by mechanisms involving reduced heme synthesis (Nayak 2002). Reduced size and numbers of erythrocytes could be associated with increased plasma volumes and decreased serum iron concentrations. However, in

order to cause serum iron to decrease by 33% (from 1.6μg/mL to 1.0μg/mL) as seen in this study, plasma volume would have to increase by 60%. This large increase in plasma volume seems unrealistic and, therefore, we are left to conclude that aluminum intake inhibits non-heme iron absorption causing reduced serum and tissue iron contents.

The mechanism by which aluminum might inhibit non-heme iron absorption is not known. The fact that serum iron was not reduced when high iron plus aluminum diets were fed suggests that high dietary iron can overcome the inhibitory effects of aluminum on iron absorption. This could result from competition between the metals for binding to iron transporters or oxidation/reduction enzymes in enterocytes that are required in the iron absorption process. Previous studies indicate that dietary aluminum reduces the amount of iron that accumulates within intestinal mucosal cells when a high iron diet is fed (Han et al. 2000). This suggests that aluminum prevents dietary iron from entering the enterocytes, and may indicate that aluminum competes with iron for uptake by the brush border divalent metal ion transporter 1 (DMT 1) or access to the ferric reductase needed to convert Fe^{+3} to Fe^{+2} prior to DMT 1 transport. Alternatively, high Al concentrations in the enterocytes may damage or down regulate components of the iron transport system (e.g., DMT1). High iron diets may mitigate this effect because high levels of iron in the intestinal lumen or a higher body iron status may inhibit the intestinal absorption of aluminum (Cannata et al. 1991) and prevent aluminum from reaching toxic concentrations in the enterocytes that would disrupt iron absorption.

Our hypothesis that aluminum reduces iron absorption is consistent with other studies where reduced tissue iron levels were found in experimental animals fed aluminum. Nasiadek and Chmieknicka (2000) observed a decrease in iron concentrations

in the spleen, erythrocytes, and blood of rats orally administered $AlCl_3$ and $FeCl_2$ together. Ecelbarger and Greger (1991) reported a 40% reduction in apparent iron absorption in rats fed aluminum hydroxide In further support of our view, Alvarez-Hernandez et al. (1994) found that aluminum loading of intestinal-derived Caco-2 cells inhibited iron transport, and Kooistra et al. (1998) reported ^{59}Fe absorption was reduced in iron-deficient hemodialysis patients when co-ingested with aluminum hydroxide. Not all studies, however, have found that oral aluminum reduces tissue iron levels (Morgan & Redgrave 1998 and Turgut et al. 2004). This may be due to the amount or species of aluminum compound used as well as the form of iron in the diet. In our studies, relatively high levels (0.3% Al) of a soluble inorganic form of aluminum ($AlCl_3$) were fed with ferric iron. The high ratio of Al^{+3}: Fe^{+3} in our diets may promote AlxFe interactions in the intestine more than lower ratios, or organically bound forms of aluminum (e.g., aluminum citrate), or when ferrous or heme iron constitute the iron source. In addition, our studies were done in rapidly growing chicks which may put a larger demand on iron absorption to supply tissue iron than in adult or slower growing animals. Further studies are needed to determine the mechanisms and extent of aluminum's potential to inhibit intestinal iron absorption.

In contrast to studies using oral aluminum administration, aluminum toxicity produced by intraperitoneal injections has been shown to increase tissue iron levels (Abreo et al. 1994 and Ward et al. 2001) and reduce serum iron (Farina et al. 2002). These effects are consistent with aluminum-loaded cell culture studies that show increased cellular uptake of transferrin-bound iron due to increased transferrin receptor expression (Abreo et al. 1994, 1999, Perez et al. 2001, and Yamanaka et al. 1999).

Therefore, it appears that the route of aluminum exposure influences aluminum's effect on tissue iron content. In the absence of dietary aluminum-induced reductions in intestinal iron absorption and serum iron concentrations, intraperitoneal injections of aluminum may enhance cellular iron uptake from the serum possibly by increased transferrin receptor expression, as supported by cell culture studies.

In addition to aluminum decreasing iron concentrations in the kidney, we observed that the aluminum-treated control and intermediate Fe groups had heavier kidneys. The composition of the kidney weight, however, was not greatly effected by aluminum since the distribution of weight among the subcellular fractions was similar among groups. How aluminum can increase the weight of the kidney is not known. The kidney weight in the high Fe + Al group was not significantly different from the nontreated groups, nor was kidney iron concentration. This may suggest that normalizing iron concentration, or limiting aluminum absorption by high iron intakes, could prevent aluminum-induced increases in kidney weight. Gomez et al. (1997) also found that aluminum intake increased kidney weight in rats, but the mechanism for this effect was not discussed.

Although neither dietary iron nor aluminum greatly altered the subcellular composition of the kidney, they did effect the accumulation of iron in these fractions. As dietary iron intake and kidney iron increased, the concentration of iron in the debris fraction and supernatant increased several fold. Much smaller increases were found in mitochondrial iron. Adding aluminum to the diets significantly increased the rate of accumulation of iron in the debris pellet, but had no effect on supernatant or mitochondrial iron accumulation (Figure 2). The lack of an effect of aluminum on

supernatant and mitochondrial iron concentrations have implications for the regulation of ferritin protein synthesis and will be discussed below. The accumulation of iron in the debris pellet may imply increased uptake by nuclei, a major component of this fraction, but our data do not indicate where this additional iron came from since both supernatant iron and iron in the mitochondrial fraction were not reduced by aluminum. However, accumulation of iron in the debris pellet agrees, in part, with results from cell culture studies. Aluminum-loaded erythroleukemia cells exposed to ^{59}Fe-transferrin showed increased accumulation of ^{59}Fe in both the nuclear and mitochondrial fractions (Abreo et al. 1994).

The accumulation of iron in the debris pellet could increase the risk of oxidative stress in this subcellular fraction. The debris pellet contains unbroken cells and nuclei. The nuclei produce the important information molecules DNA and RNA. Reactive or "free" iron that is not sequestered by ferritin, or safely bound to its normal cellular components, can increase the risk of oxidative stress. Reactive ferrous iron may catalyze the production of superoxide radicals from oxygen, and hence hydrogen peroxide via the action of superoxide dismutase. Free iron also promotes the Fenton reaction in which ferrous iron reacts with hydrogen peroxide to generate ferric iron and the hydroxyl free radical. The hydroxyl free radical is a highly reactive species which can attack nearby DNA and RNA producing modifications (Meneghini 1988). These modifications may effect gene transcription and reduce mRNA abundance. Major products of oxidative damage to DNA are 8-hydroxyguanine and 8-oxo-7,8-dihydro-2'-deoxyguanosine (Chatgilialoglu and O'Neill 2001, and Cheng et al. 1992). These free radical induced

DNA modifications have been shown to be mutagenic (Cheng et al. 1992). Oxidative damage due to increased iron in the debris pellet could be hazardous to the cell.

Our results also indicate that aluminum increases the concentration of unsequestered, reactive iron in the supernatant (i.e., cytosolic) fraction. This occurred because aluminum strongly inhibited iron uptake and storage in cytosolic ferritin. As kidney iron increased with added iron in the diet, a much smaller proportion of that iron was bound by ferritin in the aluminum treated groups (Figure 5). Since aluminum had no effect on supernatant iron concentrations, a decrease of iron bound within cytosolic ferritin would result in increased amounts of unsequestered iron in the cytosolic fraction. Cell culture studies support this conclusion. Abreo et al. (2004) showed that aluminum increased the "labile" or chelatable iron pool in cultured hepatocytes and that this was associated with increased oxidative stress. This group also showed reduced ^{59}Fe uptake into ferritin in aluminum-treated erythroleukemia cells, again accompanied by increased lipid peroxidation (Abreo et al. 1994). The only other study to address the issue of iron uptake into ferritin is the in vitro study of Fleming and Joshi (1991) who showed reduced uptake of iron into purified apo-ferritin preincubated with aluminum. Our study, then, is the first to demonstrate, in vivo, that aluminum can inhibit iron sequestration by ferritin.

Little is known about how aluminum can impair iron sequestration by ferritin. Small amounts of aluminum have been shown to bind to ferritin (Sakamoto et al. 2004). The in vitro study mentioned above (Fleming and Joshi 1991) suggested that aluminum does not affect iron release from ferritin, but affects the loading of iron into the ferritin molecule. The author's proposed that aluminum interferes with oxidation of Fe^{+2} to Fe^{+3} needed to load iron into ferritin as $Fe_2O_3 \bullet H_2O$. Abreo et al. (1994) suggested that bound

aluminum could change the conformation of ferritin resulting in impaired oxidation of Fe^{+2}. Aluminum may also obstruct or plug the pores in ferritin that allow entry of ferrous iron into the protein's core. Iron uptake into ferritin has been shown to be blocked by a chromium analogue of hydrated Fe^{+2} (Barnes et al. 2002). The chromium analogue was of similar size, shape, and charge as $[Fe(H_2O)_6]^{+2}$ and appeared to bind to negatively charged groups at the pore's entrance. Aluminum and iron have similar ionic radis, form hexahydrates, and bind to the same iron binding sites in transferrin. It is tempting to speculate that, as with Cr^{+++}, a form of Al^{+++} may bind to and block the entrance to pores in ferritin.

In addition to impaired uptake of iron into ferritin molecules, cell culture studies have shown that aluminum decreases the amount of ferritin protein. Yamanaka et al. (1999) demonstrated that aluminum increases IRP2 binding to the iron response elements of ferritin mRNA, inhibiting ferritin synthesis. They postulated that aluminum stabilized IRP2 by competing with iron for binding sites on IPR2, thereby preventing iron-induced oxidation and degradation of the regulatory protein. Alternatively, Abreo et al. (1994) proposed that aluminum caused cellular iron to be partitioned into parts of the cell (possibly mitochondria or nuclei) that are not sensed by IRPs. In this situation IRP2 would be stabilized, as if it were in an iron deficient cell, leading to down regulation of ferritin synthesis.

In vivo, however, we did not find that aluminum reduced ferritin protein levels (Figure 4). In regard to Abreo et al's. (1994) hypothesis, we were unable to show that aluminum caused iron to be partitioned out of the cytosol (the cell compartment where cytosolic IRPs presumably sense cellular iron content). Rather, we found that aluminum

had no effect on the total concentration of iron in the supernatant. Aluminum did, however, reduce the amount of supernatant iron sequestered within ferritin. This unsequestered iron in the supernatant should have increased the amount of reactive iron available to destabilize IRP2, and increase ferritin protein synthesis. The fact that ferritin protein levels were not increased may indicate that aluminum counteracted the effects of unsequestered iron, possibly as Yamanaka et al. (1999) proposed, by competing with iron for binding to IRPs. Our in vivo results, then, may be consistent with Yamanaka's hypothesis. Because the ratio of aluminum to iron within cells may be higher in cell culture studies than in vivo, this may explain the different responses seen between these experimental designs. In cell culture, aluminum levels may be high enough to enhance the stabilization of IRP2 to the point where ferritin synthesis becomes reduced.

CONCLUSIONS AND SIGNIFICANCE

The high levels of dietary aluminum used in this study (0.3g Al/100g diet) significantly reduced body iron status, mostly likely by inhibiting iron absorption. This level of aluminum intake is at the upper range of human intakes e.g., individuals consuming excessive amounts of aluminum containing antacids who may ingest 1-5g of the metal daily. It is unlikely that the relatively small intakes of aluminum in typical diets (<125mg/day) have a significant effect on iron absorption (Greger and Baier 1983).

Our study is the first to show that dietary aluminum can inhibit iron uptake into ferritin. Since the sequestration of reactive iron is an important antioxidant function of ferritin, inhibition of this protective function may be a mechanism of aluminum toxicity. We do not know at what level of dietary aluminum exposure inhibition of iron uptake by

ferritin occurs. Since aluminum may accumulate in tissues with age (Yokel and McNamara 2001) even typical exposures may eventually disrupt ferritin function. It may be that it takes only a few aluminum ions bound to ferritin to disrupt iron sequestration.

In contrast to cell culture studies, we did not find that dietary aluminum reduced ferritin protein levels. However, we believe our data support the conclusion that aluminum can stabilize IRP2 and down regulate ferritin synthesis (Yamanaka et al. 1999). The magnitude of this effect, however, may depend on the ratio of aluminum to reactive iron in the cell. Under the physiological conditions used here, kidney aluminum may not have been high enough to reduce ferritin levels below that in the control groups.

Lastly, it is interesting to speculate that the effects of aluminum on iron metabolism reflect an interaction at iron binding sites. Aluminum is known to bind to iron binding sites in transferrin and is postulated to compete with iron for binding to IRP2. In our study, competition with iron for binding to ferric reductase in the intestine or transport by DMT1 could explain reduced iron absorption and tissues iron levels. The binding of aluminum to iron sites on ferritin (e.g., entrance pores) could explain reduced iron sequestration. If this interaction with iron is a common mechanism of aluminum toxicity it could have broader implications by interfering with other aspects of iron utilization in biological systems.

FUTURE STUDY

In this study we found that high dietary aluminum suppressed tissue iron levels possibly by inhibiting intestinal iron absorption. We also showed that aluminum inhibited iron uptake and storage in ferritin without inhibiting ferritin protein synthesis

and increased the rate of accumulation of iron in the debris pellet. This suggests that aluminum may increase the concentration of unsequestered, reactive iron in the cytosol and possibly nuclei. Based on these findings, future studies in animal models or in cell cultures may focus on these aspects to further understand the effect of aluminum on iron metabolism.

There are many possible aspects that need to be examined in order to understand the effect of aluminum on intestinal absorption. The effects of Al on the expression of DMT1, ferric reductase, and ferroportin 1 in the intestine could be done at the mRNA and protein level. Aluminum can bind to iron binding sites such as transferrin. This indicates that aluminum may bind to DMT1, ferric reductase, and ferroportin 1. The binding of aluminum to these molecules could inhibit iron absorption via the competition of aluminum with iron on these sites or the alteration of these molecules by aluminum. Competition studies in cell culture systems designed to monitor iron absorption could be done. Also, interference with the newly discovered regulator for iron absorption, hepcidin, is another possible mechanism that might decrease intestinal iron absorption by aluminum. Al could increase production and release of hepcidin by the liver. The release of hepcidin can then modify the crypt cells to produce a low level of duodenal ferric reductase, ferroportin 1, and DMT1. Therefore, the interaction of aluminum with DMT1, ferroportin, ferric reductase, and hepcidin are all possible mechanisms that can have an affect on intestinal iron absorption.

Decreased uptake of iron by ferritin due to aluminum could be studied by testing the mechanism of incorporation of iron into ferritin. Ferrioxidase enzymes are located along the ferritin pores to oxidize Fe^{+2} to the stored form of iron within the ferritin core.

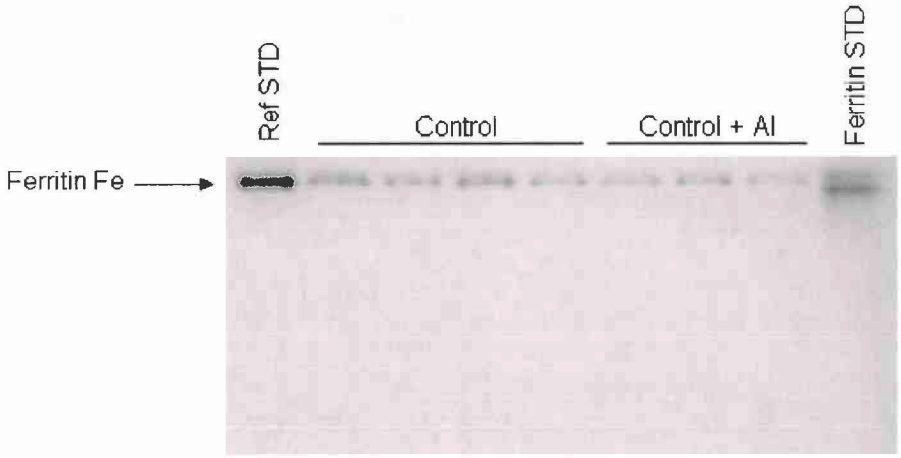
Its activity might change due to the toxicity of aluminum resulting in the inhibition of iron uptake into ferritin. Besides the enzyme activities, aluminum may obstruct or plug the pores in ferritin that allow entry of ferrous iron into the protein's core. The site of Al binding to ferritin needs to be identified.

Yamanaka et al. (1999) demonstrated that aluminum increases IRP2 binding to the iron response elements of ferritin and transferrin receptor mRNA, inhibiting ferritin synthesis and increasing transferrin receptor. However, the level of ferritin protein remained unchanged in our study. Our study was conducted using live animals and Yamanaka's study was in cell cultures, it is possible that in vivo and in vitro may have different responses due to different concentrations of Al in cells. In this respect it would be helpful to measure Al and free iron levels. Also, to understand what is really going on, the level of tranferrin receptor, ferritin protein, and the mRNA for transferrin receptor, and ferritin need to be examined in vivo.

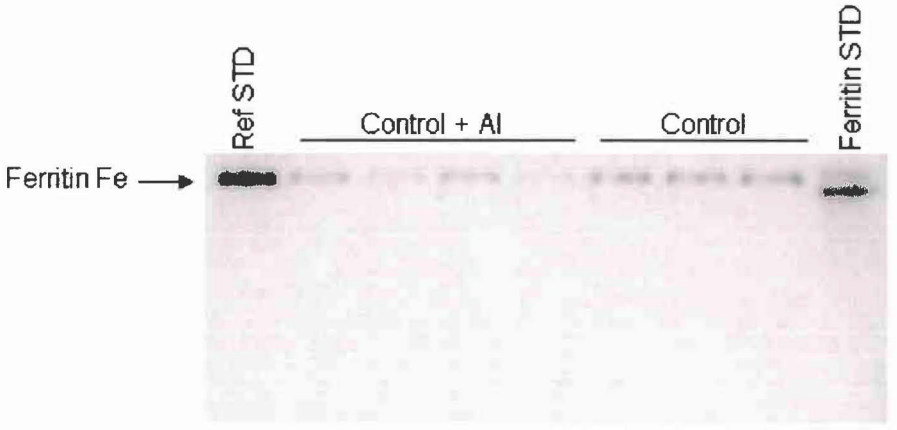
The accumulation of iron in the debris pellet and increased of unsequestered iron in the cytosol might increase the risk of oxidative stress to DNA and cells. In the future, studies need to be conducted to investigate if there is an increase in reactive iron and oxidative stress in these fractions. To measure the relationship of an increased iron to oxidative damage, oxidative products such as malondialdehyde, and 8-hydroxyguanine, and 7,8-dihydro-8-oxo-2'-deoxyguanosine could be tested in tissues.

Appendix A. Native PAGE gel assays for ferritin bound iron in kidney supernatants.

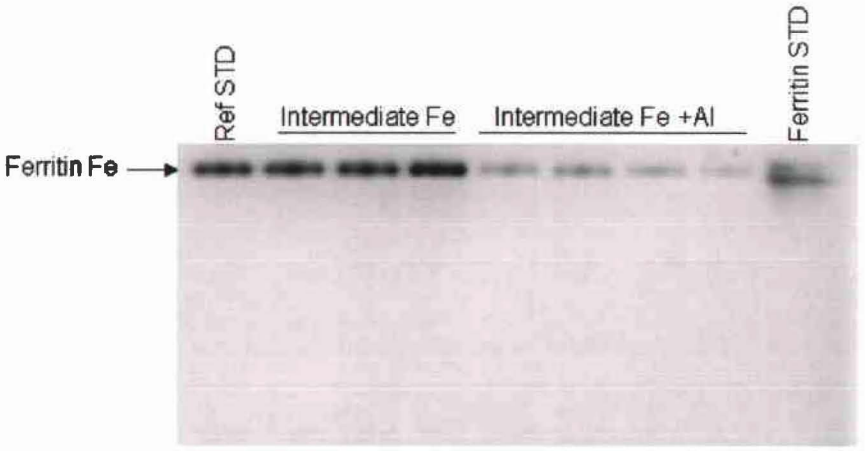
Figures 7-12 illustrate the native-PAGE gels stained for iron from experiments I and II. A horse spleen ferritin standard (2 μg) was loaded along with samples to identify the location of ferritin on the gel. Ferritin bound iron band intensities were expressed as a ratio to the band intensity of the reference sample (R1) present on all gels. Also shown for each sample are the concentrations of iron in both the kidney and kidney supernatant fractions. Figures 7-8: Heat stable proteins (100 μg) from kidney supernatant of the control group (samples #1-4 & 12-14) and control + Al group (samples #5-11). Figures 9-10: Heat stable proteins (100 μg) from kidney supernatant of the intermediate iron group (samples #1-2 & 4) and intermediate iron + Al group (samples #5-16). Figures 11-12: Heat stable proteins (40 μg) from kidney supernatant of the high Fe group (samples #1-4 & 9-12) and high Fe + Al group (samples #5-8 & 13-16).



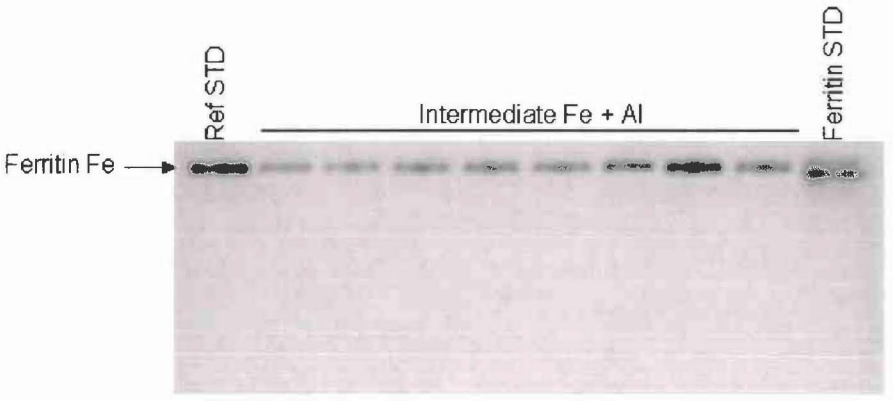
Sample #:	R1	1	2	3	4	5	6	7
Kidney Fe (µg/g kidney):	65	56	49	48	41	40	42	38
Supernatant Fe (µg/g supnt):	2.8	2.5	1.7	1.9	1.5	1.6	1.5	1.6
Fe band intensity (ratio to R1):	1	0.37	0.23	0.33	0.17	0.13	0.16	0.12



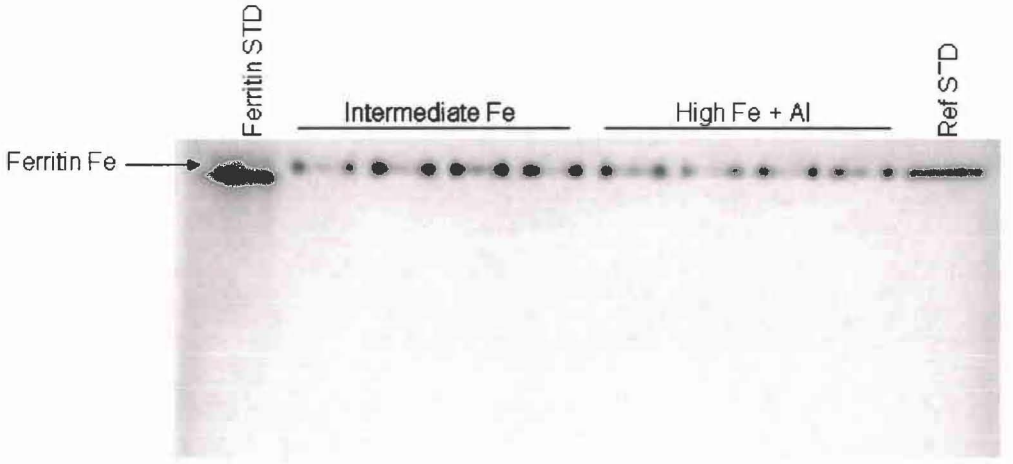
Sample # :	R1	8	9	10	11	12	13	14
Kidney Fe (µg/g kidney):	65	50	31	36	31	46	48	45
Supernatant Fe (µg/g supnt):	2.8	2.0	1.3	1.6	1.3	2.0	1.8	2.0
Fe band intensity (ratio to R1):	1	0.22	0.09	0.2	0.08	0.28	0.28	0.32



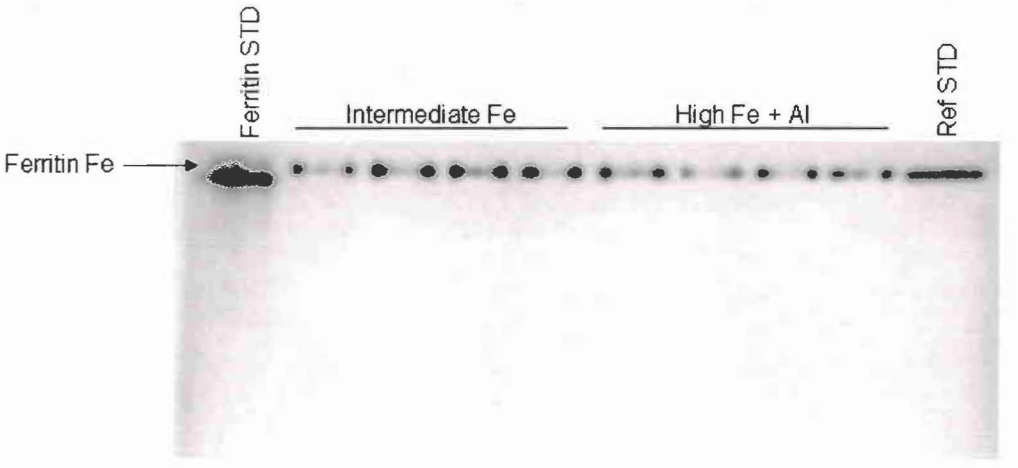
Lane:	R1	1	2	4	5	6	7	8
Kidney Fe (µg/g kidney):	65	58	60	61	39	36	29	30
Supernatant Fe (µg/g supnt):	2.8	2.4	2.3	2.5	1.1	1.0	1.0	0.9
Fe band intensity (ratio to R1):	1	1.08	1.15	1.32	0.37	0.39	0.26	0.15



Sample #:	R1	9	10	11	12	13	14	15	16
Kidney Fe (µg/g kidney):	65	31	32	43	36	32	31	38	36
Supernatant Fe (µg/g supnt):	2.8	1.0	1.0	1.1	1.2	1.0	1.1	1.6	1.1
Fe band intensity (ratio to R1):	1	0.31	0.33	0.43	0.49	0.46	0.51	0.98	0.55



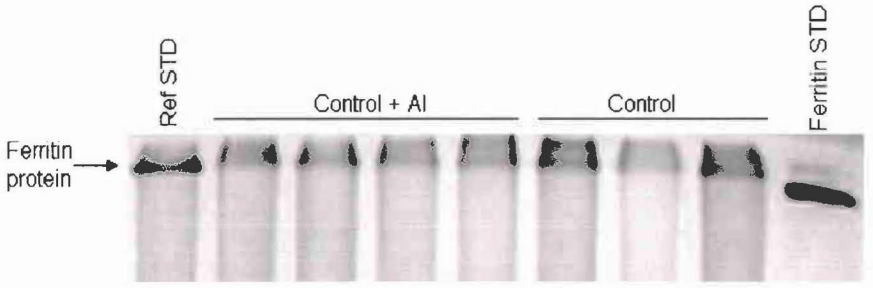
Sample # :	1	2	3	4	5	6	7	8	R1
Kidney Fe (µg/g kidney)	47	59	54	69	52	55	53	63	65
Supernatant Fe (µg/g supnt)	1.9	2.5	2.3	2.3	1.8	2.0	2.4	2.3	2.8
Fe band intensity (ratio to R1)	0.56	0.89	0.86	0.74	0.72	0.44	0.56	0.59	1



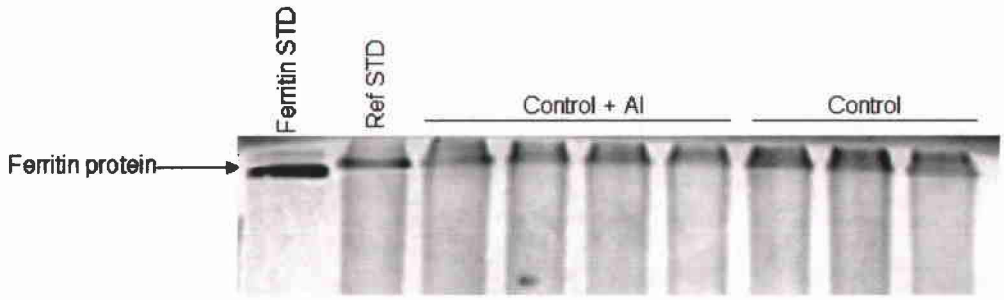
Sample # :	1	2	3	4	5	6	7	8	R1
Kidney Fe (µg/g kidney):	47	59	54	69	52	55	53	63	65
Supernatant Fe (µg/g supnt)	1.9	2.5	2.3	2.3	1.8	2.0	2.4	2.3	2.8
Fe band intensity (ratio to R1)	0.56	0.89	0.86	0.74	0.72	0.44	0.56	0.59	1

Appendix B. Native PAGE gel assays for ferritin protein in kidney supernatants.

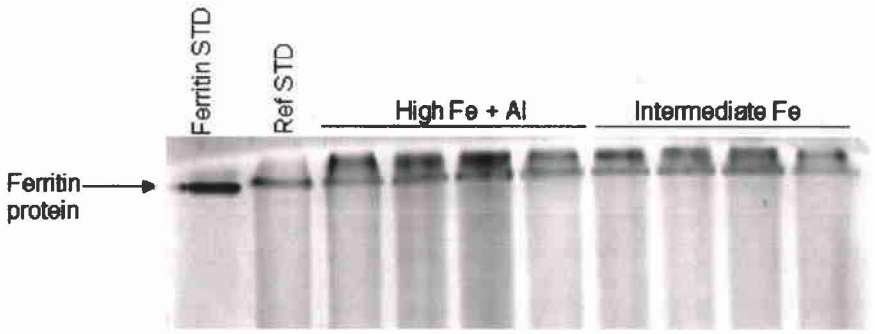
Figures 13-18 illustrate the native-PAGE gels from experiments I and II stained for ferritin protein. A horse spleen ferritin standard (5 μg) was loaded to identify the location of ferritin protein on the gel. Ferritin band intensities are express as a ratio to the band intensity of the reference sample (R2). Also shown for each sample are the concentrations of iron in both the kidney and kidney supernatant fractions, and the ratio of ferritin iron to ferritin protein. Figures 13-14: Heat stable proteins (10 μg) from kidney supernatant of the control group (samples #1-4 & 12-14) and control + Al group (samples #5-11). Figures 15-16: Heat stable proteins (10 μg) from kidney supernatant. Samples #1-4 & 9-12 were from the intermediate Fe group and samples #5, 7-8 & 13-16 were from the high Fe plus aluminum group. Figure 17-18: Heat stable proteins (10 μg) from kidney supernatant. Samples #1 & 3-4 were from the intermediate iron group and samples #5-16 were from the intermediate iron plus aluminum group.



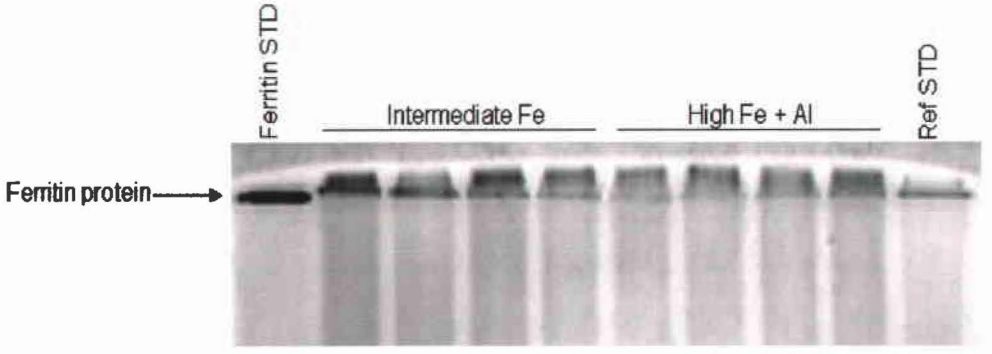
Sample # :	R2	7	6	5	4	3	2	1
Kidney Fe (µg Fe/g kidney):	60	38	42	40	41	48	49	56
Supernatant Fe (µg Fe/g supnt):	2.3	1.6	1.5	1.6	1.5	1.9	1.7	2.5
Protein band intensity (ratio to R2):	1	0.36	0.48	0.51	0.62	0.54	0.35	0.68
Ratio ferritin Fe : ferritin protein:	1	0.33	0.33	0.25	0.27	0.61	0.66	0.5



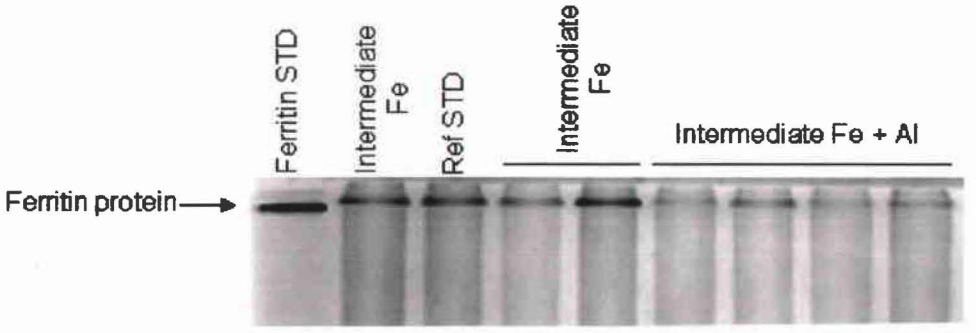
Sample #:	R2	8	9	10	11	12	13	14
Kidney Fe (µg Fe/g kidney):	60	50	31	36	31	46	48	45
Supernatant Fe (µg Fe/g supnt):	2.3	2.0	1.3	1.6	1.3	2.0	1.8	2.0
Protein band intensity (ratio to R2):	1	0.53	0.56	0.57	0.59	0.71	0.79	0.82
Ratio ferritin Fe : ferritin protein:	1	0.42	0.16	0.35	0.14	0.39	0.35	0.39



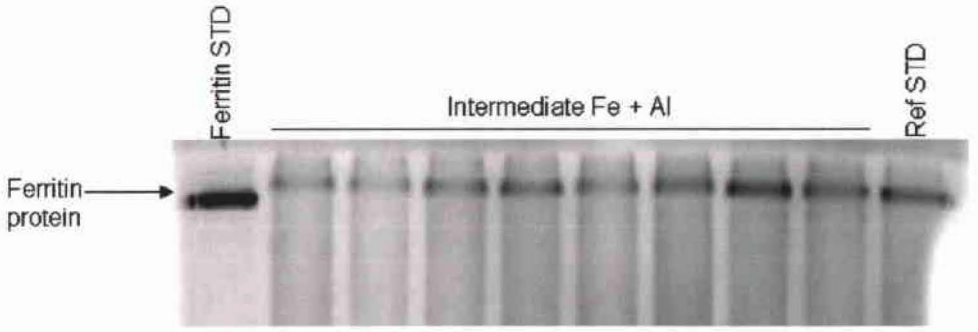
Sample # :	R2	8	7	6	5	4	3	2	1
Kidney Fe (µg Fe/g kidney):	60	63	53	63	52	69	54	59	47
Supernatant Fe (µg Fe/g supnt):	2.3	2.3	2.4	2.3	1.8	2.3	2.3	2.5	1.9
Protein band intensity (ratio to R2):	1	1.11	1.01	1.11	0.97	0.83	0.73	1.02	0.92
Ratio ferritin Fe : ferritin protein:	1	0.53	0.55	0.53	0.74	0.89	1.18	0.87	0.61



Sample #:	9	10	11	12	13	14	15	16	R2
Kidney Fe (µg Fe/g kidney):	59	60	51	48	48	43	46	49	60
Supernatant Fe (µg Fe/g supnt):	2.6	1.7	2.3	2.4	1.7	1.7	1.9	2.0	2.3
Protein band intensity (ratio to R2):	1.54	1.41	1.14	1.03	0.93	1	1.01	0.79	1
Ratio ferritin Fe : ferritin protein:	0.62	0.76	0.73	0.8	0.35	0.31	0.27	0.32	1



Sample #:	1	R2	3	4	5	6	7	8
Kidney Fe (µg Fe/g kidney):	58	60	65	61	39	36	29	30
Supernatant Fe (µg Fe/g supnt):	2.4	2.3	2.8	2.5	1.1	1.0	1.0	0.9
Protein band intensity (ratio to R2):	1.05	1	0.85	1.01	0.55	0.63	0.54	0.53
Ratio ferritin Fe : ferritin protein:	1.03	1.15	1.18	1.31	0.67	0.62	0.48	0.28



Sample # :	16	15	14	13	12	11	10	9	R2
Kidney Fe (µg Fe/g kidney):	36	38	31	32	36	43	32	31	60
Supernatant Fe (µg Fe/g supnt):	1.1	1.6	1.1	1.0	1.2	1.1	1.0	1.0	2.3
Protein band intensity (ratio to R2):	0.46	0.45	0.67	0.75	0.58	0.73	1.07	0.94	1
Ratio ferritin Fe . ferritin protein.	1.2	2.18	0.76	0.61	0.84	0.59	0.31	0.33	1

REFERENCE

Abboud, S. and Haile, D.J. (2000) A Novel Mammalian Iron-Regulated Protein Involved in Intracellular Iron Metabolism. The Journal of Biological Chemistry 275(926):19906-19912.

Abreo, K., Glass, J., and Sella, M. (1990) Aluminum Inhibits Hemoglobin Synthesis but Enhances Iron Uptake in Friend Erythroleukemia Cells. Kidney International 37:677-681.

Abreo, K., Jangula, J., Jain, S., and Glass, J. (1991) Aluminum Uptake and Toxicity in Cultured Mouse Hepatocytes. Journal of the American Society of Nephrology 1:1299-1304.

Abreo, K. and Glass, J. (1993) Cellular, Biochemical, and Molecular Mechanisms of Aluminum Toxicity. Nephrology Dialysis Transplantation 1:5-11.

Abreo, K., Glass, J., Jain, S., and Sella, M. (1994) Aluminum Alters the Compartmentalization of Iron in Friend Erythroleukemia Cells. Kidney International 44:636-41.

Abreo, K., Abreo, F., Sella, M., and Jain, S. (1999) Aluminum Enhances Iron Uptake and Expression of Neurofibrillary Tangle Protein in Neuroblastoma Cells. Journal of Neurochemistry 72:2059-2064.

Abreo, K., Sella, M., Alvarez-Hernandez, X., and Jain, S. (2004) Antioxidants Prevent aluminum-Induced Toxicity in Cultured Hepatocytes. Journal of Inorganic Biochemistry 98:1129-134.

Adzersen, K.H., Becker, N., Steindorf, K., and Frentzel-Beyme, R. (2003) Cancer Mortality in a Cohort of Male German Iron Foundry Workers. American Journal of Industrial Medicine 43:295-305.

Aisen, P., Enns, C., and Wessling-Resnick, M. (2001) Review: Chemistry and Biology of Eukaryotic Iron Metabolism. The International Journal of Biochemistry and Cell Biology 33:940-959.

Alfrey, A. C. (1991) Aluminum Intoxication, Recognition and Treatment. In: Aluminum in Chemistry, Biology and Medicine, pp73-84. Cortina International, Verona.

Alfrey, A.C., leGendre, G.R. and Kaehny, W.D. (1976) The Dialysis Encephalopathy Syndrome-Possible Aluminum Intoxication. New England Journal of Medicine 294:184-188.

Ali, M.A., Akhmedkhanov, A., Zeleniuch-Jaquotte, A., Toniolo, P., Frenkel, K., and Huang, X. (2003) Reliability of Serum Iron, Ferritin, Nitrite, and Association with Risk of Renal Cancer in Women. Cancer Detection and Prevention 27:116-121.

Alvarez-Hernandez, X., Madigosky, S.R., Stewart, B., and Glass, J. (1994) Iron Status Affects Aluminum Uptake and Transport by Caco-2 Cells. Journal of Nutrition 124:1574-1580.

Arosio, P. and Levi, S. (2002) Ferritin, Iron Homeostasis, and Oxidative Damage. Free Radical Biology and Mecidine 33:457-63.

Bali, P.K., Zak, O., and Aisen, P. (1991) A New Role for the Transferrin Receptor in the Release of Iron from Transferrin. Biochemistry 30:324-328.

Barnes, C.M., Theil, E.C., and Raymond, K.N. (2002) Iron Uptake in Ferritin is Blocked by Binding of $[Cr(TREN)(H_2O)(OH)]^{2+}$, A Slow Dissociating Model for $[Fe(H_2O)_6]^{2+}$. Proceedings of the National Academy of Sciences 99(8):5195-5200.

Bartzokis, G. (2004) Brain Ferritin Iron as a Risk Factor for Age at Onset in Neurodegenerative Diseases. Annals of the New York Academy of Sciences 1012:224-36.

Bhasin, G., Kauser, H., Sarwar, A.M. and Athar, M. (2003) Progressive Iron Overload Enhances Chemically Mediated Tumor Promotion in Murine Skin. Archives of Biochemsitry and Biophysics. 409:262-73.

Bondy, S.C. and Kirstein, S. (1996) The Promotion of Iron-Induced Generation of Reactive Oxygen Species in Nerve Tissue by Aluminum. Molecular and Chemical Neuropathology 2:185-94.

Borenstein, A., White, E., Koepsell, T.D., Reifler, B.V., Van Belle, G., and Larson, E.B. (1990) The Association Between Aluminum-Containing Products and Alzheimer's Disease. Journal of Clinical Epidemiology 43:35-44.

Bourdon, E., Kang, D.K., Ghosh, M.C., and Drake, S.K. (2003) The Role of Endogenous Heme Synthesis and Degradation Domain Cysteines in Cellular Iron-Dependent Degradation of IRP2. Blood Cells, Molecules, and Diseases 31:247-255.

Bourgoin, B.P. (1992) Alumino-Silicate Content in Calcium Supplements Derived from Various Carbonate Deposits. Bullutin of Environmental Contamination Toxicology 48:803-8.

Bradbear, R.A., Bain, C., and Siskind, V. (1985) Cohort Study of Internal Malignancy in Genetic Hemochromatosis and Other Chronic Non-Alcoholic Liver Diseases. Journal of the National Cancer Institute 75:81-84.

Broe, G.A., Henderson, A.S., Creasey, H., McCusker, E., Korten, A.E., Jorm, A.F., Longley, and Anthony, J.C. (1990) A Case-Control Study of Alzheimer's Disease in Australia. Neurology 40:1698-1707.

Calado, R.T., Franco, R.F., Pazin, A. (2000) HFE Gene Mutations in Coronary Atherothrombotic Disease. Brazilian Jouranl of Medical Biological Research 33:301-306.

Candore, G., Balistreri, C.R., Lio, D., Mantovani, V., Colonna-Romano, G., Chiappelli, M., Tampieri, C., Licastro, F., Branzi, A., Verna, M., Caruso, M., Hoffmann, E., and Caruso, C. (2003) Association Between HFE Mutations and Acute Myocardial Infarction: A Study in Patients from Northern and Southern Italy. Blood Cells, Molecules, and Diseases 31:57-62.

Cannata, J.B., Gomez, Alonso, C., Fernandez, Menendez, M.J., Fernandez, Soto, I., McGregor, S., Menendez-Fraga, P., and Brock, J.H. (1991) Iron Uptake in Aluminum Overload: In Vivo and In Vitro Studies. Nephrology Dialysis Transplantation 6(9):637-42.

Cederbaum, A.I. (2003) Iron and CYP2E1-Dependent Oxidative Stress and Toxicity. Alcohol 30:115-120.

Chatgilialoglu, C. and O'Neill, P. (2001) Free Radicals Associated with DNA Damage. Experimental Gerontology 36:1459-1471.

Chau, L.Y. (2000) Iron and Atherosclerosis. Proceedings of the National Science Council, Republic of China. Part B, Life Sciences 24(4) 151-155.

Chen, S.Y. Hayes, R.B., Liang, S.R., Li, Q.G.. Stewart, P.A., and Blair, A. (1990) Mortality Experience of Haematite Mine Workers in China. British Journal of Industrial Medicine 47:175-181.

Cheng, K.C., Cahill, D.S., Kasai, H., Nishimura, S., and Loeb, L.A. (1992) 8-Hydroxyguanine, an Abundant Form of Oxidative DNA Damage, Causes G→ T and A→ C Substitutions. Journal of Biological Chemistry 267:166-172.

Chung, M.C.-M. (1985) A Specific Iron Stain for Iron-Binding Proteins in Polyacrylamide Gels: Application to Transferrin and Lactoferrin. Analytical Biochemistry 148:498-502.

Clauberg, M. and Joshi, J.G. (1993) Regulation of Serine Protease Activity by Aluminum: Implications for Alzheimer's Disease. Proceedings of the National Academy Sciences 90:1009-1012.

Conrad, M.E., Umbreit, J.N., Moore, E.G., Nainsworth, L.N. Porubcin, M., Simovich, M.J., Nakada, M.T., Dolan, K., and Garrick, M.D. (2000) Separate Pathways for Cellular Uptake of Ferric and Ferrous Iron. American Journal of Physiology: Gastrointestinal Liver Physiology 279:G767-G774.

Corsi, B., Cozzi, A., Arosio, P., Drysdale, J., Santombrogio, P., and Campanellas, A. (2002) Human Mitochondrial Ferritin Expressed in HeLa Cells Incorporates Iron and Affects Cellular Iron Metabolism. The Journal of Biological Chemistry 277:22430-22437.

Crichton, R.R. (1991) Chapter 6: Chemistry and Biology of the Transferrins. In: Inorganice Biochemistry of Iron Metabolism, pp101-119. Ellis Horwood Limited, Chichester, West Sussex, England.

Crichton, R.R. (1971) Medical Progress. Ferritin: Structure, Synthesis and Function. The New England Journal of Medicine 284:1413-1422.

Crichton, R.R. (2001) Inorganice Biochemistry of Iron Metabolism. In: Molecular Mechanisms to Clinical Consequences, 2nd Edition. Wiley, Chichester, New York.

Crichton, R.R., Wilmet, S., Legssyer, R., and Ward, R.J. (2002) Molecular and Cellular Mechanisms of Iron Homeostasis and Toxicity in Mammalian Cells. Journal of Inorganic Biochemistry 91(1):9-18.

Davis, M.T. and Bartfay, W.J. (2004) Ebselen Decreases Oxygen Free Radical Production and Iron Concentrations in the Hearts of Chronically Iron-Overloaded Mice. Biological Research For Nursing 6(1):37-45.

Deng, Z., Coudray, C., Gouzoux, L., Mazur, A., Royssiguier, Y., and Pepin, D. (2000) Effects of Acute and Chronic Coingestion of $AlCl_3$ with Citrate or Polypheolic Acids on Tissue Retention and Distribution of Aluminum in Rats. Biological Trace Element Research 76:245-256.

Deugnier, Y. (2003) Iron and Liver Cancer. Alcohol 30:145-150.

D'Haese, P.C and De Broe, M.E. (2001) Chapter 11: Aluminum and Iron: Implications for Alzheimer's Disease. In: Aluminum and Alzheimer's Disease the Science that Describes the Link, pp221-231. Elsevier Science B.V., Amsterdam, Netherlands.

Domingo, J.L. (1993) Aluminum: Toxicology. In: Encyclopedia of Food Science, Food Techonology and Nutrition, vol. 1, 130-53. Academic Press, London.

Drueke, T.B. (2002) Intestinal Absorption of Aluminum in Renal Failure. Nephrology Dialysis Transplantation 17(2):13-16.

Dunn, M.A., Johnson, N.E., Lieu, M.Y., and Ross, E. (1993) Dietary Aluminum Chloride Reduces the Amount of Intestinal Calbindin D-28K in Chicks Fed Low Calcium or Low Phosphorus Diets. Journal of Nutrition 123(11):1786-93.

Ecelbarger, C.A. and Greger, J.L. (1991) Dietary Citrate and Kidney Function Affect Aluminum, Zinc and Iron Utilization in Rats. Journal Nutrition 121:1755-1762.

Eden T. (1976) Tea. Pp8-15. Longman, London.

Edwardson, J.A., Ferrier, I.N., McArthur, F.K., McKeith, I.G., McLaughlin, I., Morris, C.M., Mountfort, S.A., Oakley, A.E., Taylor, G.A., Ward, M.D., and Candy, J.M. (1991) Alzheimer's Disease and the Aluminum Hypothesis. In: Aluminum in Chemistry, Biology and Medicine, pp84-96. Raven Press, New York.

Emerit, J., Beaumont, D., and Trivin, F. (2001) Iron Metabolism, Free Radicals, and Oxidative Injury. Biomedicine Pharmocotherapy 55(6)333-339.

Exley, C. (2001) Preface: Why is Research into Aluminum and Life Important? In: Aluminum and Alzheimer's Disease The Science that Describes the Link, pp V-VIII. Elsevier, Amsterdam, The Netherlands.

Farina, M., Lara, P.S., Brandao, R., Jacques, R., Rocha, J.B.T. (2002) Effects of Aluminum Sulfate on Erythropoiesis in Rats. Toxicology Letters 132:131-9.

Fatemi, S.J., and Moore, G.R. (1991) Aluminum Transport in Blood Serum. Binding of Aluminum by Human Transferrin in the Presence of Human Albumin and Citrate. Biochemical Journal 280:527-32.

Ferguson, C.J., Wareing, M., Ward, D.T., Green,R., Smith, C.P., and Riccardi, D. (2001) Cellular Localization of Divalent Metal Transporter DMT-1 in Rat Kidney. American Journal Physiology-Renal Physiology 280:F803-F814.

Fleming, J.T. and Joshi, J.G. (1991) Ferritin: The Role of Aluminum in Ferritin Function. Neurobiology of Again. 12:413-418.

Francanzani, A.L., Conte, D., Fraquelli, M., Taioli, E., Mattioli, M., Losco, A., and Fargion, S. (2001) Increased Cancer Risk in a Cohort of 230 Patients with Hereditary hemochromatosis in Comparison to Matched Control Patients with Non-Iron Related Chronic Liver Disease. Hepathology 33:647-651.

Franco, R.F. Zago, M.A., Trip, M.D. (1998) Prevalence of Hereditary Hemochromatosis in Premature Atherosclerotic Vascular Disease. British Journal Haematology 102:1172-1175.

Gaenzer, H., Marschang, P., Sturm, W., Neumayr, G., Vogel, W., Patsch, J., Weiss, G. (2002) Journal of the American College of Cardiology 40:2189-2194.

Ganrot, P.O. (1986) Metabolism and Possible Health Effects of Aluminum. Environmental Health Perspectives 65:363-441.

Golub, M.S., Han, B., and Keen, C.L. (1996) Aluminum Alters Iron and Manganese Uptake and Regulation of Surface Transferrin Receptors in Primary Rat Oligodendrocyte Cultures. Brain Research 719:72-77.

Gomez, M., Sanchez, D.J., Llobet, J.M., Corbella, J., & Domingo, J.L. (1997) The Effect of Age on Aluminum Retention in Rats. Toxicology 116:1-8.

Gonzalez-Revalderia, J., Casares, M., de Paula, M., Pasucual, T., Giner, V., and Miravalles, E. (2000) Biochemical and Hematological Changes in Low-Level Aluminum Intoxication. Clinical Chemistry and Laboratory Medicine 38(3):221-5.

Greger, J.L. (1993) Aluminum Metabolism. Annual Review Nutrition 13:43-63.

Greger, J.L and Sutherland, J.E. (1997) Aluminum Exposure and Metabolism. Critical Reviews in Clinical Laboratory Sciences 34(5): 439-474.

Greger, J.L. and Baier, M.J. (1983) Effect of Dietary Aluminum on Mineral Metabolism of Adult Males. The American Journal of Clinical Nutrition 38:411-419.

Groff, J.L. and Gropper, S.S. (2000) Chapter 12: Microminerals. In: Advanced Nutrition and Human Metabolism 3rd Edition, pp402-417. Wadsworth, Belmont, CA.

Gurzau, E.S. Corneliu, N., and Gurzau, A.E. (2003) Essential Metals-Case Study on Iron. Ecotoxicology and Environmental Safety 56:190-200.

Guthrie, H.A. and Picciano, M.F. (1995) Chapter 10: Micronutrient Minerals. In: Human Nutrition, pp334-351. Mosby, St. Louis, Missouri.

Han, J., Han, J., and Dunn, M.A. (2000) Effect of Dietary Aluminum on Tissue Nonheme Iron and Ferritin Levels in the Chick. Toxicology 142: 97-109.

Harley, A., Cooper, J.M., Schapira, A.H. (1993) Iron Induced Oxidative Stress and Mitochondrial Dysfunction: Relevance to Parkinson's Disease. Brain Research 627(2): 349-53.

Hentze, M.W., Muchenthaler, M.U., and Andrews, N.C. (2004) Balancing Acts: Molecular Control of Mammalian Iron Metabolism. Cell 117:285-297.

Hetet, G., Elbaz, A., Gariepy, J. (2001) Association Studies Between Haemochromatosis Gene Mutations and the Risk of Cardiovascular Diseases. European Journal of Clinical Investigation 31: 382-388.

Hsing, A.W., McLaughlin, J.K., Olsen, J.H., Mellemkjar, L., Wacholder, S., Fraumeni, J.F.J. (1995) Cancer Risk Following Primary Hemochromatosis: A Population-Based Cohort Study in Denmark. International Journal of Cancer 60: 160-162.

Hubert, N., and Hentze, M.W. (2002) Previously Uncharacterized Isoforms of Divalent Metal Transporter (DMT1): Implications for Regulation and Cellular Function. Proceedings of the National Academy of Sciences 99:12345-12350.

Iribarren, C., Sempos, C.T., Eckfeldt, J.H., and Folsom, A.R. (1998) Lack of Association Between Ferritin Level and Measured of LDL Oxidation: The ARIC Study. Atherosclerosis Risk in Communities. Atherosclerosis 139:189-95.

Jacobs, A. (1975) Iron Deficiency: Effects on Tissues and Enzymes In: Iron Metabolism and Its Disorders, pp135-145. Exerpta Medica, Amsterdam, Oxford.

Jang, J.H., and Surh, Y.J. (2002) Beta-Amyloid Induces Oxidative DNA Damage and Cell Death Through Activation of c-Jun N Terminal Kinase. Annals of the New York Academy of Sciences 973: 228-36.

Jansson, E.T. (2001) Aluminum Exposure and Alzheimer's Disease. Journal of Alzheimer's Disease 3: 541-549.

Jeong, S.Y., and David, S. (2003) Glycosylphosphatidylinositol-Anchored Ceruloplasmin is Required for Iron Efflux from Cells in the Central Nervous System. Journal of Biological Chemistry 278:27144-27148.

Kaiser, L., Schwartz, K., Burnatowska-Hledin, M.A., and Mayor G. (1984) Microcytic Anemia Secondary to Intraperitoneal Aluminum in Normal and Uremic Rats. Kidney International 26: 269-274.

Kausz, A.T., Antonsen, J.E., Hercz, G., Pei, Y., Weiss, N.S., Emerson, S., and Sherrard, D.J. (1999) Screening Plasma Aluminum Levels in Relation to Aluminum Bone Disease among asymptomatic Dialysis Patients. American Journal Kidney Diseases 34: 688-693.

Ke, Y., Chen, Y.Y., Chang, Y.Z., Duan, X.L., Ho, K.P., Jiang, D.H., Wang, K., and Qian, Z.M. (2003) Post-Transcriptional Expression of DMT1 in the Heart of Rat. Journal of Cellular Physiology 196:124-130.

Kooistra, M.P., Niemantsverdriet, E.C., van Es, A., Mol-Beer-mann, N.M., Struyvenberg, A., Marx, J.J. (1998) Iron Absorption in Erythropoietin-treated Haemodialysis patients: Effects of Iron Availability, Inflammation and Aluminum. Nephrology, Dialysis, Transplantation 13:82-88.

Lash, L.H. and Sall, J.M. (1993) Chapter 1: Mitochondrial Isolation from Liver and Kidney: Strategy, Techniques, and Criteria for Purity. In: Methods in Toxicology, pp8-28. Academic Press. Inc., San Diego, California.

Layrisse, M. (1975) Dietary Iron Absorption. In: Iron Metabolism and Its Disorders, pp25-34. Exerpta Medica, Amsterdam, Oxford.

Le, N.T.V. and Richardson, D.R.R. (2002) Ferroportin 1: A New Iron Export Molecule? The International Journal of Biochemistry & Cell Biology 34: 103-108.

Leong, W.I., and Lonnerdal, B. (2004) Hepcidin, the Recently Identified Peptide that Appears to Regulate Iron Absorption. 134:1-4.

Lemmer, E.R., Gelderblom, W.C.A., Shephard, E.G., Abel, S., Seymour, B.L., Cruse, J.P., Kirsch, R.E., Marasas, W.F.O., and Hall, P.M. (1999) The Effects of Dietary Iron Overload on Fumonisin B1-Induced Cancer Promotion in the Rat Liver. Cancer Letters 146:207-215.

Lieu, P.T., Heiskala, M. Peterson, P.A., and Yang, Y. (2001) The Roles of Iron in Health and Disease. Molecular Aspects of Medicine 22(1-2)1-87.

Martinet, W., Knaapen, M.W.M., De Meyer, G.R.Y. (2002) Elevated Levels of Oxidative DNA Damage and DNA Repair Enzymes in Human Atherosclerotic Plaques. Circulation 106:927-932.

McGregor, S.J., Naves, M.L., Birly, A.K., Russell, N.H., Halls, D., Junor, B.J., and Brock, J.H. (1991) Interaction of Aluminum and Gallium with Human Lymphocytes: The Role of Transferrin. Biochimica et Biophysica ACTA/General Subjects 1095:196-200.

McKie, A.T., Marciani, P., Rolfs, A., Bernnan, K., Wehr, K., Barrow, D., Miret, S., Bomford, A., Peters, T.J., Farzaeh, F., Hediger, M.A., Hentze, M.W., and Simpson, R.J. (2000) A Novel Duodenal Iron-Regulated Transporter, IREG1, Implicated in the Basolateral Transfer of Iron to the Circulation. Molecular Cell 5:299-309.

McLachlan, D.R.C., Bergeron, C., Smith, J.E., Boomer, D., and Rifat, S.L. (1996) Risk for Neuropathologically Confirmed Alzheimer's Disease and Residual Aluminum in Municipal Drinking Water Employing Weighted Residential Histories. Neurology 46: 401-405.

Meneghini, R. (1988) Genotoxicity of Active Oxygen Species in Mammalian Cells. Mutation Research/DNA Repair 195:215-230.

Milman, N., Pedersen, P., Steig, T., Byg, K., Graudal, N., Fenger, K. (2001) Clinically Overt Hereditary Hemochromatosis in Denmark 1948-1985: Epidemiology, Factors of Significance for Long-Term Survival, and Causes of Death in 179 patients. Annals of Hematology 80:737-744.

Moore, M., Folsom, A.R., Barnes, R.W., and Eckfeldt, J.H. (1995) No Association Between Serum Ferritin and Asymptomatic Carotid Atherosclerosis. The Atherosclerosis Risk in Communities (ARIC) Study. American Journal Epidemiology 141(8):719-23.

Morgan, E.H. and Redgrave, T.G. (1998) Effects of Dietary Supplementation with Aluminum and Citrate on Iron Metabolism in the Rat. Biological Trace Element Research 65:117-131.

Nasiadek, M. and Chmielnicka, J. (2000) Interaction of Aluminum with Exogenous and Endogenous Iron in the Organism of Rats. Ecotoxicology and Environmental Safety. 45: 284-290.

Nayak, P. (2002) Aluminum: Impacts and Disease. Environmental Research Section A 89: 101-115.

Nelson, R.L. (2001) Iron and Colorectal Cancer Risk: Human Studies. Nutrition Review 59:140-148.

Nesse, A. and Garbossa, G. (2001) Chapter 13: Aluminum Toxicity in Erythropoiesis. Mechanisms Related to Cellular Dysfunction in Alzheimer's Disease. In: Aluminum and Alzheimer's Disease. The Science that Describes the Link, pp261-277. Elsevier Science B.V., Amsterdam, The Netherlands.

Ng, M. (2004) Role of Hydrogen Peroxide in the Aetiology of Alzheimer's Disease: Implications for Treatment. Drugs Aging 21(2):81-100.

Nicolas,G., Bennoun, M., Devaux, I., Beaumont, C., Grandchamp, B., Kahn, A., and Vaulont, S. (2001) Lack of Hepcidin Gene Expression and Severe Tissue Iron Overload in Upstream Stimulatory Factor 2 (USF2) Knockout Mice. Proceedings National Academy of Sciences 98:8780-8785.

Niederau, C., Fischer, R., Sonnenberg, A., Stremmel, W., Trampisch, H.J., Strohmyer, G. (1985) Survival and Causes of Death in Cirrhotic and in Noncirrhotic Patients with Primary Hemochromatosis. New England Journal of Medicine 313: 1256-1262.

Orino, K., Harada, S., Natsuhori, M., Takehara, K., and Watanabe, K. (2004) Kinetic Analysis of Bovine Spleen Apoferritin and Recombinant H and L Chain Homopolymers: Iron Uptake Suggests Early Stage H Chain Ferroxidase Activity and Second Stage L Chain Cooperation. BioMetals 17(2):129-34.

Oshiro, S., Kawahara, M., Mika, S., Muramoto, K., Kobayashi, K, Ishige, R., Nozawa, K., Hori, M., Yung, C., Kitajima, S., & Kuroda, Y. (1998) Aluminum Taken Up by Transferrin-Independent Iron Uptake Affects the Iron Metabolism in Rat Cortical Cells. Journal of Biochemistry 123:42-46.

Oteiza, P.I., Fraga, C.G., and Keen, C.L. (1993) Aluminum Has Both Oxidant and Antioxidant Effects in Mouse Brain Membranes. Archives of Biochemistry and Biophysics 300: 517-521.

Perez, G., Garbossa, G., and Nesse, A. (2001) Disturbance of Cellular Iron Uptake and Utilisation by Aluminum. Journal of Inorganic Biochemistry 87:21-27.

Pham, Q.T., Teculescu, D., Bruant, A., Chau, N., Viaggi, M.N., and Rebstock, E. (1992) Iron Miners-a 10 Year Follow-Up. European Journal of Epidemiology 8:594-600.

Picard, V., Epsztejn, S., Santambrogio, P., Cabantchik, Z.I., and Beaumont, C. (1998) Role of Ferritin in the Control of the Labile Iron Pool in Murine Erthroleukemia Cells. Journal of Biological Chemistry 273(25):15382-15386.

Pratico, D., Uryu, K., Sung, S., Tang, S., Trojanowski, J.Q., and Lee, V.M.Y. (2002) Aluminum Modulates Brain Amyloidosis through Oxidative Stress in APP Transgenic Mice. The Federation of American Societies for Experimental Biology Journal 16(9):1138-40.

Rasmussen, M.L., Folsom, A.R., Catellier, D.J. (2001) A Prospective Study of Coronary Heart Disease and the Hemochromatosis Gene (HFE) C282Y Mutation: The Atherosclerosis Risk in Communities (ARIC) Study. Atherosclerosis 154:739-746.

Robson, K.J., Lehmann, D.J., Wimhurst, V.L., Livesey, K.J., Combrinck, M., Merryweather-Clarke, A.T., Warden, D.R., and Smith, A.D. (2004) Synergy Between the C2 Allele of Transferrin and the C282Y Allele of the Haemochromatosis gene (HFE) as Risk Factors for Developing Alzheimer's Disease. Journal of Medical Genetics 41(4):261-5.

Roest, M., van der Schouw, Y.T., and de Valk, B. (1999) Heterozygosity for a Hereditary with Cardiovascular Death in Women. Circulation 100:1268-1273.

Rodrigues, M., Felsenfeld, A.J., and Lalch, F. (1990) Aluminum Administration in the Rat Separately Affects the Osteoblast and Bone Mineralization. Journal of Bone Mineral Research 5: 59-67.

Rondeau, V., Commenges, D., Jacqmin-Gadda, H., and Dartigues, J.F. (2000) Relation between Aluminum Concentrations in Drinking Water and Alzheimer's Disease: An 8-year Follow-up Study. American Journal of Epidemiology 152(1): 59-66.

Roy, A., Solodovnikova, N., Nicholson, T., Anotholine, W., and Walden, W.E. (2003) A Novel Eukaryotic Factor for Cytosolic Fe-S Cluster Assembly. The European Molecular Biology Organization Journal 22: 4826-4835.

Sakamoto, T., Ogasawa, Y., Ishii, K., Takahashi, H., & Tanabe, S. (2004) Accumulation of Aluminum in Ferritin Isolated from Rat Brain. Neuroscience Letter 366(3):264-7.

San-Marina, S., and Nicholls, D.M. (1992) Some Effects of Aluminum on Rat Brain Protein Synthesis. Comparative Biochemistry and Physiology 103(3):585-591.

Shaheen, N.J., Silverman, L.M., Keku, T., Lawrence, L.B., Rohlfs, E.M., Martin, C.F., Galanko, J., and Sandler, R.S. (2003) Association Between Hemochromatosis (HFE) Gene Mutation Carrier Status and the Risk of Colon Cancer. Journal of the National Cancer Institute 95: 154-9.

Skalsky, M.L., and Carchman R.A. (1983) Aluminum Homeostasis in Man. Jouranl of the American College Toxicology 2: 405-423.

Sipe, D.M. and Murphy, R.F. (1991) Binding to Cellular Receptors results in Increased Iron Release from Transferrin at Mildly Acidic pH. Journal of Biological Chemistry 266: 8002-8007.

Soldado, Cabezuelo, A.B., Blancogonzalez, E., and Sanz Medel, A. (1997) Quantitative Studies of Aluminum Binding Species in Human Uremic Serum by Fast Protein Liquid Chromatography with Electrothermal Atomic Absorption. Analyst 122: 573-77.

Stevens, R.G., Jones, D.Y., Micozzi, M.S. and Taylor, R.R. (1988) Body Iron Stores and the Risk of Cancer. New England Journal of Medicine 319:1047-1052.

Takeuchi, K., Okada, S., Yukihiro, S., and Inoue, H. (1997) The Inhibitory Effects of Aluminum and Fe on Bone Formation, in Vivo and in Vitro Study. Pathophysiology 4: 97-104.

Taylor, G.A., et al. (1995) Alzheimer's Disease and the Relationship between Silicon and Aluminum in Water Supplies in Northern England. Journal of Epidemiology Community Health 49: 323-324.

Toyokuni, S. (2002) Iron and Carcinogenesis: From Fenton Reaction to Target Genes. Redox Report 7: 189-97.

Tuomainen, T.P., Diczfalusy, U., Kaikkonen, J., Nyyssonen, K., and Solenen, J.T. (2003) Serum Ferritin Concentration is Associated With Plasma Levels of Cholesterol Oxidation Products in Man. Free Radical Biology & Medicine 35:922-928.

Tuomainen, T.P., Kontula, K., Nyyssonen, K. (1999) Increased Risk of Acute Myocardial Infarction in Carriers of the Hemochromatosis Gene Cys282Tyr Mutation: A Prospective Cohort Study in Men in Eastern Finland. Circulation 100: 1274-1279.

Turgut, G., Kaptonaglu, B., Turgut, S., Enli, Y., and Genc, O. (2004) Effects of Chronic Aluminum Administration on Blood and Liver Iron-Related Parameters in Mice. Yonsei Medical Journal 45(1):135-139.

Uc, A., Strokes, J.B., and Britigan, B.E. (2004) Heme Transport Exhibit Polarity in CaCo-2 Cells: Evidence for an Active and Membrane Protein-Mediated Process. American Journal of Physiology: Gastrointestinal Liver Physiology Doi:10.1152/ajpgi.00157.2004

U.S. Public Health Service. (1992) "Toxicological Profile for Aluminum & Compounds." (Contact No. 205-88-0608). Prepared by Clement International Corp.

Van der Voet, G.B. (1992) Intestinal Absorption of Aluminum. In: Aluminum in Biology and Medicine. (Ciba Foundation Symposium 169), pp107-17. John Wiley and Sons, Chichester.

Varner, J.A., Jensen, K.F., Horvath, W., Isaacson, R.L. (1998) Chronic Administration of Aluminum-Fluoride or Sodium-Fluoride to Rats in Drinking Water: Alterations in Neuronal and Cerebravascular Integrity. Brain Research. 784: 284-298.

Venturini-Soriano, M., and Berthon, G. (2001) Aluminum speciation studies in biological fluids. Part 7. A Quantitative Investigation of Aluminum (III)-Malate Complex Equilibria and their Potential Implications for Aluminum Metabolism and Toxicity. Journal of Inorganic Biochemistry 85: 143-154.

Van Rensburg, S.J., Daniels, W.M., Potocnik, F.C., Van Zyl, J.M., Taljaard, J.J., Emsley, R.A. (1997) A New Model for the Pathophysiology of Alzheimer's Disease. Aluminum Toxicity is Exacerbated by Hydrogen Peroxide and Attenuated by An Amyloid Protein Fragment and Melatonin. South Africa Medical Journal 87(9): 1111-5.

Waalen, J., Felitti, V., Gelbart, T., Ho, N.J., and Beutler, E. (2002) American Journal of Medicine 113:472-479.

Walter, P.B., Knutson, M.D., Paler-Martinez, A., Lee, S., Xu, Y., Viteri, F.E., and Ames, B.N. (2002) Iron Deficiency and Iron Excess Damage Mitochondria and Mitochondrial DNA in Rats. Proceedings of the National Academy Sciences. 99(4):2264-9.

Ward, R.J., Zhang, Y., and Crichton, R.R. (2001) Aluminum Toxicity and Iron Homeostasis. Journal of Inorganic Biochemistry 87: 9-14.

Whittaker, P. and Chanderbhan, R.F. (2001) Effect of Increasing Iron Supplementation on Blood Lipids in Rats. British Journal of Nutrition 86: 1-11.

Xie, C.X., Mattson, M.P., Lovell, M.A., and Yokel, R.A. (1996) Intraneuronal Aluminum Potentiates Iron-Induced Oxidative Stress in Cultured Rat Hippocampal Neurons. Brain Research. 743:271-277.

Xu, Z., Pan, G.W., Liu, L.M., Brown, L.M., Guan, D.X., Xiu, Q., Sheng, J.H., Stone, B.J., Dosemeci, M., Fraumeni Jr, J.F., and Blot, W.J. (1996) Cancer Risks Among Iron and Steel Workers in Anshan, China. Part I. Proportional Mortality Ratio Analysis. American Journal of Industrial Medicine 30:1-6.

Yamanaka, K., Minato, N., Iwai, K. (1999) Stabilization of iron regulatory protein 2, IRP2, by aluminum. Federation of European Biochemical Societies Letters 462: 216-220.

Yokel, R.A., Allen, D.D., and Ackley, D.C. (1999) The Distribution of Aluminum Into and Out of the Brain. Journal of Inorganic Biochemistry 76: 127-132.

Yokel, R. and McNamara, P.J. (2001) Aluminum Toxicokinetics: An Updated MiniReview. Pharmacology & Toxicology 88: 159-167.

Youdim, M.B., Ben-Shachar, D., Riederer, P. (1993) The Possible Role of Iron in the Etiolpathology of Parkinson's Disease. Movement Disorders 8(1): 1-12.

Zatta, P., Taylor, A., Zambenedetti, P., Milacic, R. and dell'Antone, P. (2000) Aluminum Inhibits the Lysosomal Proton Pump from Rat Liver. Life Sciences 66:2261-2266.