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Studies of select trace element nutrition upon cardiac electrical, morphometrical and ultrastructural aspects of the rat and pig

Wildman, Robert E. C., Ph.D.
The Ohio State University, 1994
STUDIES OF SELECT TRACE ELEMENT NUTRITION UPON
CARDIAC ELECTRICAL, MORPHOMETRICAL AND ULTRASTRUCTURAL
ASPECTS OF THE RAT AND PIG

DISSERTATION

By

Robert E.C. Wildman, B.S., M.S., R.D.

* * * * *

The Ohio State University
1994

Dissertation Committee:
D.M. Medeiros
R.L. Hamlin
J.D. Bonagura

Approved by

Advisor

College of Human Ecology
Department of Human Nutrition
and Food Management
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VITA

November 19, 1964 ................... Born - Philadelphia, PA

1988 ............................. B.S., University of Pittsburgh
                              Pittsburgh, PA

1991 ............................. M.S., The Florida State
                              University, Tallahassee, FL

1991-1992 ......................... Graduate Teaching Associate
                              Department of Human Nutrition
                              and Food Management,
                              The Ohio State University
                              Columbus, Ohio

1992-1993 ......................... Graduate Research Associate
                              Department of Human Nutrition
                              and Food Management,
                              The Ohio State University
                              Columbus, Ohio

1993-1994 ......................... Helen Lapp Fellowship
                              Recipient, Department of Human
                              Nutrition and Food Management,
                              The Ohio State University
                              Columbus, Ohio

1992-1994 ......................... Adjunct Faculty, Columbus
                              State Community College,
                              Columbus, OH

FIELD OF STUDY

Major Field: Human Ecology

                   Department of Human Nutrition and Food Management

Minor Field: Cardiac Physiology
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CHAPTER I
INTRODUCTION

Once considered the soul of the human being, the heart has since been more accurately recognized as a muscular pump. Blood is pumped throughout the human body by means of ventricular contraction and the subsequent establishment of a pressure gradient within the systemic and pulmonic circulations (1). Oxygenated blood returning from the lungs and deoxygenated blood returning from the systemic circulation, empty into the left and right atria, respectively, and drain into the ventricles. The left ventricle pumps oxygen rich blood to all tissue in the body, while, the right ventricle pumps blood to the lungs for pulmonary respiration gaseous exchange with the outside environment.

Cardiac muscle is excitable tissue. It responds to an electrical stimulus by rapidly and transiently changing its membrane potential. The electrical signal is a wave of electronegativity, originating in cardiac autorhythmic cells, that traverses the heart. Voltage-gated ion channels, including Ca^{2+} channels, open and dramatically increase the intracellular Ca^{2+} concentration and evoke cardiomyocyte sarcomere shortening.
Due to the rich electrolyte concentration of body fluids, electrical impulses generated at and traversing the heart are transmitted outward. Utilizing electrodes (leads), cardiac electrical impulses can be registered, amplified, and recorded at torso and extremity surfaces as electrocardiograms (ECGs). ECGs allow insight not only to the timing, magnitude, and direction of electrical impulses emanating from the heart, but also provides an evaluative basis for cardiac pathology.

At the onset of the cardiac cycle, oxygenated and deoxygenated blood passively drain from the atria, into the left and right ventricles, respectively. After the period of passive ventricular filling, the wave of electrical stimulation traverses the atria and incites contraction. Atrial contraction pumps additional blood into the ventricles.

The electrical stimulus typically originates within the autorhythmic cells of the SA (SinoAtrial) node, which is often referred to as the "pacemaker" of the heart (1). The wave of electrical activation originating in the SA node moves across the atria in a right to left fashion, while concomitantly and more expeditiously, it commutes from the SA node to the AV (AtrioVentricular) node over specialized internodal conductive tissue (1). The depolarization of the atrial myocytes gives rise to the P-wave on a typical ECG. The quantity of P waves per unit time yields insight to the number of times per minute an electrical signal normally negotiates the heart in efforts to manifest cardiac contraction (beats per minute, b.p.m.).
The wave of activation carried on the internodal conductive tissue crosses the AV node on route to the ventricles. It is the AV node that retards the velocity of the electrical signal, due to conductile fiber diameter reduction, and allows for a momentary pause between atrial and ventricular contraction. The electrical signal then traverses the Bundle of His and the left and right main bundles on route to the apical region of the heart. The electrical signal ultimately reaches the ends of the specialized Purkinje fibers shocking the ventricular myocytes into contraction (1). Activation is then carried out in a myocyte to myocyte fashion due to the presence of gap junctions at intercalated discs. Ventricular myocytes, so organized as to wring the ventricles in an apical to basal fashion, contract, ejecting blood into a "great artery" (aortic or pulmonary). The QRS complex of the ECG represents the electrical depolarization of ventricular tissue. Thus, the PQ (PR) interval signifies the elapsed time between the onset of atrial depolarization to the beginning of ventricular depolarization (1).

Internal ventricular pressure is rapidly elevated by the ventricular contraction. As the blood is compressed, the rising ventricular pressure quickly overcomes the intra-atrial pressures. This results in the closure of the mitral and tricuspid AV valves. Intraventricular pressure continues to rise during isovolumetric contraction until it overcomes the pressures in the aorta and pulmonic trunk. Once these
pressures are overcome, the semilunar valves open and blood is propelled into these great vessels. During the ventricular contraction time interval the developed pressure (dP) may be measured. The dP/dt (developed pressure/difference in time) is considered an indicator of ventricular contractility. Furthermore, the dP/dt_max is a measure of the maximum ascent in ventricular pressure per unit time. The dP/dt_max is affected not only by myocardial contractility but also preload (End Diastolic Volume (EDV)) in accordance to the Frank-Starling law of the heart (1). Finally, the QRS complex is followed momentarily by the T wave, which represents the repolarization of the ventricular tissue (1).

The P wave, QRS complex and the T wave are the typical features of the ECG and alterations in magnitude, appearance, and interval lengths between the waves elicit noninvasive information regarding the electrical characteristics of the heart. Contrarily, dP/dt measurement requires an invasive approach. ECGs and developed pressure measurements are by no means the only assessment techniques of cardiology. Echocardiology utilizes a transducer emitting ultrasonic waves (high frequency) into the thorax which may be manipulated to view the heart from different perspectives. As the ultrasonic waves reach the heart and related structures, the waves are reflected back whenever a boundary of density differential is encountered (regions of varied acoustical impedances). The reflected sound waves, or cardiac "echoes", are received by
the transducer and displayed in the form of a cardiac image on a oscilloscope or strip chart recorder.

The heart may be excised and preserved for examination of the gross morphological aspects of cardiac structures. Heart tissue may also be fixated, magnified and examined utilizing light or electron microscopy. These procedures allow for visual examination of the organ at the gross, tissue, cellular and subcellular levels. Since clinical symptomology is often a reflection of aberrations at the tissue cell level, combining all of the previously mentioned procedures can yield a greater understanding of a given pathology. Other physiological and clinical biochemical indices are available the allow insight to cardiac health. These include blood enzyme levels, radiographic techniques, phonocardiography, cardiac angiography, and techniques of cardiac imaging (ie. magnetic resonance imaging).

The study of cardiovascular health and longevity is of primary concern in United States, a country where heart disease is the leading cause of mortality (2). Non inherited factors suggested to augment the probability, or risk, of heart disease include a sedentary lifestyle (2,3), smoking (2,3) and a high saturated fat-high lipid diet (2-4). More tangibly the results of many controlled animal studies demonstrate that the status of certain trace elements such as Cu (5-9), Se (10-13) and Fe (14-17) may have a direct impact upon the development of cardiac complications. The focus of
this dissertation is to first examine the comparative aspects of cardiomyopathy in the rat fed either a copper, iron, or selenium restricted diet, and second, to examine copper-deficient cardiomyopathy in the pig. The first concept, involving three different element restrictions, expounds from individual studies of each of the three trace elements. This study employs a comparative perspective. The second concept, using the pig as a model, was pursued due to the greater similarity of the pig heart to the human heart.

The effects of biologically deficient minerals upon the anatomical and physiological properties of the heart are dependent on factors such as: the deficient mineral, extent of the deficiency (intake and duration of deficiency), species, age, gender, and other interacting factors such as concomitant exercise, disease, or pharmacological treatment.

Historically, copper-deficient animals have exhibited alterations in electrocardiograms (5-9). Prohaska & Heller (5) reported decreased heart rates in copper deficient rats, while Kopp and colleagues (6) did not observe a similar effect. Alterations of ECG features include abnormalities of ST segments, increased PR intervals, increased R wave duration and amplitude (5-8) and the presence of ectopic ventricular and supraventricular contractions (7). Kopp and colleagues (6) reported augmented HV intervals in the His Bundle electrocardiogram proposing decelerated electrical conduction through the His-Purkinje system. Medeiros et al (8) reported
QRS waves of greater amplitude in copper deficient animals, indicative of hypertrophy.

Morphometrical studies have shown copper-deficient cardiac hypertrophy to be of a concentric nature highly similar to human concentric heart disease attributed to pressure overload (18-20). Also, cardiac lesions at the intracellular level have been reported (6,7). Ultrastructural alterations include myocyte hypertrophy, with increased mitochondria volume density, increased mitochondria:myofibril ratio, fragmented cristae, abnormal appearance of basal laminae, pericapillary collagen fibrosis and changes at the capillary-myocyte interface (8,9).

Although extreme copper deficiency in humans has not been reported, the average daily intake of copper for men and women has been estimated below the National Research Council Recommendations (21). Apart from a marginal dietary intake of copper, other dietary factors can decrease copper bioavailability (22). Therefore investigation of copper status and organ system physiology is warranted.

The deficiency of another trace element, selenium (Se), was identified as the endemic heart disease factor in a discrete region of China (Keshan disease)(10). Keshan disease, is characterized by cardiac insufficiency, gross hypertrophy, arrhythmias and electrocardiographic alterations (10). Since the acknowledgment of selenium deficiency in Keshan disease, two other selenium-deficiency related
cardiomyopathies have been documented. Firstly, a cardiomyopathy with clinical and pathological similarities has been described in long term recipients of Total Parenteral Nutrition (TPN) (11-13). In one case study a 17 year old girl developed dilated cardiomyopathy after receiving selenium-deficient TPN over a protracted period (11). Secondly, selenium was reported as a detrimental factor in individuals with Acquired ImmunoDeficiency Syndrome (AIDS) (23). It has been observed that both pediatric and adult AIDS patients developed dilated cardiomyopathy and that the etiology is undetermined (24-26). Dworkin and colleagues (23) observed marked reductions in blood selenium levels and glutathione peroxidase activity in AIDS patients. In a follow-up study, the same group examined eight AIDS patients for selenium status at autopsy (24). They observed a significant reduction in cardiac selenium and histological examination also revealed mild hypertrophy and/or fibrosis.

Beyond copper and selenium, iron deficiency has too been associated with heart disease. Classical studies dating back before the turn of the century, and in the years that followed, stated that severe anemia resulted in cardiac hypertrophy (27-30). These observations were made in both man and experimental animals, although, the mechanism for the pathological alteration remains elusive to date. One of the proposed mechanisms of cardiac myocyte hypertrophy identifies alterations in catecholamine metabolism in iron deficiency.
Previous work demonstrated that augmented norepinephrine indeed induces cardiac hypertrophy (34-36).

The most common deficiency encountered in nutritional surveys in the United States is that of iron deficiency (37). Iron intakes below recommended levels have been associated with certain metabolic anomalies involving complexes and enzymes with iron constituency (14). The effect of iron deficiency can be realized as part of heme structures, such as hemoglobin, myoglobin and the cytochromes, or, as part of non-heme enzymes, such as succinic dehydrogenase (14). Therefore, the ramifications of iron deficiency are involved at both the mitochondrial and cytoplasmic compartments of the cell. The observations of low iron intakes in nutritional surveys together with the cardiac ramifications associated with poor iron status, substantiates the need for further investigation.

Much of the cardiovascular investigation, in regard to trace element deficiencies, employs the rat as the animal model. The rationale for this choice may often involve economic considerations, but the applicability of experimental results to human heart disease must be questioned. In adjunction, we must ask, with regard to the heart disease, whether there is a more suitable animal model for human comparison. In certain situations, pathological investigation utilizing a particular species may be inappropriate for human comparison due to one or more nonconserved anatomical or
physiological mechanisms between the two species. The rat offers many similarities to the human heart but as a comparative model it has limitations.

It is well established that an atypical lipid profile is a risk factor for certain types of heart disease (4). The rat does not share a plasma lipid profile characteristic of the human, and thus, this variable remains uncontrolled. The pig, on the other hand, offers a plasma lipid profile with greater similarity to humans and also exhibits a more conserved cardiac structuring (chamber walls, dimensions, and cardiac valves).

Statement of the Problem

Heart disease is the leading cause of mortality in the United States. Biological deficiencies of essential trace elements copper, selenium, and iron have been shown to be involved in the development of cardiac pathologies in humans and animals. These three nutritional deficiency models are believed to involve generally different etiological mechanisms for the development of their respective cardiac pathologies. Conversely, there are definite similarities and common features between the three models. Copper-deficiency leads to the development of concentric hypertrophy, presumably from myocyte enlargement with a greater mitochondrial fraction.
Iron-deficiency also yields cardiac myocyte hypertrophy, but at the gross level, the cardiac hypertrophy is of the eccentric nature, similar to that of selenium deficiency related heart complications.

Both iron and copper are constituents of complexes involved in the electron transport chain and oxidative phosphorylation. Copper is found in functional cytochrome c oxidase while iron is part of the heme containing cytochromes. Therefore, alterations in the availability of copper and iron will ultimately effect the optimal operation of nutrient oxidation in the mitochondria. This is a vital concept, as the healthy heart will obtain a substantial portion of its energy demands by the mitochondrial oxidation of fatty acids and pyruvate.

A common characteristic of both dietary iron and copper restrictions, is a reduction in blood hemoglobin concentration. Hemoglobin is the principle mechanism for oxygen transport in the mammal. Thus, a reduction in hemoglobin concentration results in a relative reduction in oxygen transport to tissue. The reduced oxygen transport may exacerbate the effects of the reduced oxidative phosphorylation mentioned above. Dietary iron restriction affects hemoglobin manufacturing when it is unavailable for heme synthesis. Conversely, dietary copper restriction effects the activity of ceruloplasmin, a copper containing ferroxidase enzyme, which ultimately limits iron’s
availability for heme synthesis again, despite an elevated liver iron concentration.

Free radical oxidation is a primary function of the antioxidant enzyme complexes, Superoxide Dismutase (SOD) and Glutathione Peroxidase. One prominent intracellular SOD enzyme complex contains Cu and previous work has shown a marked decrease in the level of enzyme activity when dietary copper is restricted. Glutathione peroxidase is a selenium containing intracellular enzyme complex (SeGSHpx) whose reported activity levels has also marked decrease when dietary selenium is restricted. Thus, restrictions in either dietary selenium or copper can effect the antioxidation properties of body tissue, including the heart.

Thus, a comparative examination of the three isolated trace element deficiencies within a controlled setting would greatly enhance our understanding of differences and similarities of their associated cardiomyopathies. Comparative parameters will include electrocardiography, since cardiac alterations at the cellular and gross levels can be identified noninvasively in the form of ECGs. Comparative evaluation will also include left ventricular dP and (+/-)dP/dt_{max} measurements, as a measure of systolic and diastolic function, and examination at the gross (morphometrical measurements) and subcellular level (electron microscopy).

In regard to copper-deficiency alone, much of the work has been completed using the rat model. Since much of the
focus of animal research is in regard to application to similar pathologies of humans, we must ask the question: Do the previous experimental findings apply outside rodentia? The pig offers a heart that is structurally and functionally more related to that of humans. Some work has been completed utilizing the pig as a model, but more investigation needs to be conducted to validate some of the clinical findings of copper-deficient cardiac myopathy as more than just a developed pathology in rats. We need to continue taking the necessary steps to apply this knowledge to similar scenarios in humans.

**Objectives of the proposed studies:**

1. Evaluate the similarities and differences of iron, selenium, and copper-restricted rat hearts. Comparisons were made between between the three mineral restricted groups and a mineral adequate group. Parameters of comparison included: electrocardiograms, dP/dts, morphometrical characteristics, and ultrastructural aspects.

2. Investigate the development and characteristics of copper-deficient pigs. Variables of investigation included: both electrocardiographic and blood parameter (hematocrit, hemoglobin, and cholesterol) evaluation at three different times during the study to assess the development of copper-
deficiency. Also, electron microscopy of cardiac myocyte ultrastructure and lipoprotein profiles were determined at the conclusion of the study.
CHAPTER II
REVIEW OF LITERATURE

The following dissertation studies utilize dietary restrictions of three different trace elements: copper, iron and selenium. The design of the two studies will allow for: 1) a comparative approach to the cardiac pathologies associated with copper, iron, and selenium diet restrictions in the rat under the same time frame (study 1), and 2) to examine the cardiac pathology and plasma lipid profiles associated with pigs fed a copper-restricted diet (study 2). Therefore a review of the literature pertaining to function of copper, selenium, and iron and their deficiency related cardiac pathologies is important.

Copper

The transition element copper (Cu) is not among the most abundant elements found in nature. Nriagu (38) estimated the copper concentration of the Earth's crust to be $15 \times 10^{17}$ kg split between tillable soils and water. The possibility of copper playing a role in the vitality of organisms was acknowledged in the early half of the 19th century (39). The essentiality of copper in regard to hematopoiesis, separate from iron, was established by Hart and colleagues in 1928.
In the years that followed, copper's essentiality in regard to enzoic neonatal ataxia prevention was also established (41). Reviews of experimental and observational data by Hoagland in 1932 (42) and Arnon and Stout in 1939 (43) served as the basis for the determination of copper's essentiality for plants and animals.

Organisms appear to maintain a fairly conserved overall copper concentration of approximately 2 μg/g (wet weight), although a few exceptions have been acknowledged (39). Within multicellular organisms, the distribution of copper varies with tissue type. In plants the highest concentration can be found within the seeds, especially in portions constituting the embryonic plant (39). In regard to animals and fowl, copper concentration is greater in the embryos and neonates (animal seeds) than measured in the adult of the specie, including the human (44,45). The studies that follow focus upon mammalian tissue, therefore the remainder of the review will address mammalian copper metabolism and deficiency pathophysiology. The review of selenium and iron that follow will also be mostly restricted to mammalian physiology and deficiency related pathophysiology.

Apart from nails and hair, the tissue types exhibiting the highest copper concentration are kidney, liver, brain, heart and skeleton while other tissue such as skin, blood and adipocytes exhibit lower amounts (39). The importance and localization of a greater skeletal tissue (including the
marrow) copper concentration has yet to be established.

Typically intracellular copper concentrations are greater than that found in body fluids with the exception of bile, which is the major excretory mechanism, and cerebral spinal fluid, for reasons not readily apparent. Organ copper concentrations remain generally consistent among vertebrates, although exceptions have been reported in dogs and sheep (39).

The human dietary intake of copper has been estimated at 0.93 mg/day for women and 1.24 mg/day for men (21). These amounts fall below the Safe and Adequate Dietary Intake for copper as established by the National Research Council in 1989 (adults 1.5-3.0 mg per day) (46). Of the ingested Cu, more than half is absorbed. Pecal copper concentration is a primarily a combination of unabsorbed dietary copper, bile copper, and enterocyte copper as a result of intestinal cell sloughing. Absorbed copper traverses the enterocyte and with some regulation crosses the serosal membrane and enters the bloodstream where it mostly combines with albumin and transcuprin. These plasma proteins serve to transport copper to the liver and kidney, the first sites of deposition. Once copper is within hepatic tissue it can either interact with bile, be incorporated in intracellular proteins or be incorporated into ceruloplasmin for secretion into the blood and delivery to target tissues (39).

Copper is utilized by most cells as a component of the enzymes cytochrome c oxidase and superoxide dismutase. Copper
is also a constituent of many other cupro-enzymes, including lysyl oxidase, involved in cross-linking of connective tissue proteins elastin and collagen fibers, dopamine ß hydroxylase (dihydroxyphenyl ethylamine ß hydroxylase), involved in the conversion of dopamine to norepinephrine (catecholamine synthesis), and ceruloplasmin, involved in copper transport, extracellular oxygen radical scavenging, and iron transport (47-49). It is believed that most of the developed cardiac pathology associated with copper-deficiency is directly attributed to alterations of the activities of these and other vital cupro-enzymes.

Weanling rats fed a copper-deficient diet for a period of 5-6 weeks develop cardiac hypertrophy and failure, and exhibit high rates of mortality due to hemothorax or aneurysms (7,50,51). Previous studies have documented that the hypertrophied heart is concentric in nature where the ventricular free walls and interventricular septum are grossly thickened and the ventricular lumen narrowed (8). This is in contrast to another form of hypertrophy, eccentric, where the overall perimeter of the heart is greater, ventricular free walls thinner, but, greater lumen volume apparent. Concentric hypertrophy may occur as a result of pressure-overload, such as the conditions of systemic, aortal hypertension and aortic stenosis or idiopathic hypertensive cardiomyopathy. Systemic hypertension in the copper-deficient rat has been observed but is an inconsistent finding and not essential for the
development of hypertrophy (52). Concentric hypertrophy differs structurally from eccentric hypertrophy which is often referred to as volume-overload. Eccentric hypertrophy may occur in such conditions such as anemia and/or fluid retention where the volume of blood pumped per unit time is increased to meet metabolic demands (31).

Concentric hypertrophy does occur in humans and is often thought to have a genetic basis. The copper-deficient rat heart may serve as a useful model to study concentric hypertrophy in the absence of hypertensive etiology. Mitral and tricuspid AV valves are compromised in the copper-deficient rat heart (51), however, if the compromised valve structure in copper-deficiency played a major role in the cardiac hypertrophy an eccentric pattern would be expected in contrast to the observed concentric pattern. Regurgitation across incompetent atrioventricular valves during ventricular systole would establish a volume overload scenario (1).

The effects of dietary copper restriction are made apparent by alterations in cupro-enzyme activities and tissue Cu concentration. Reductions in cytochrome c oxidase (54,55), Cu,Zn SOD (9,56,57) and ceruloplasmin (5) are consistent findings along with reduced Cu concentration of organs (5,6,55,56,58) and blood (58,59) and are used to gage the severity of biological copper deficiency. Of the several key cuproenzymes in the heart, cytochrome c oxidase has been studied most by our laboratory. Cytochrome c oxidase (CCO) is
associated with the mitochondrial inner membrane and catalyzes the transfer of electrons from ferricytochrome c to oxygen (60). Therefore, the copper containing cytochrome c oxidase is essential in mitochondrial electron transfer and oxidative phosphorylation. CCO is actually a enzyme complex consisting of several nuclear and mitochondrial encoded subunits (60). Recent findings suggested that the nuclear encoded subunits of this enzyme are markedly depressed in the copper-deficient rat heart, but not the mitochondrial encoded-subunits (56). CCO subunits fail to complex into an active CCO enzyme in the absence of any of the individual subunits. Thus, a decrease in CCO activity may result in the reduction of mitochondrial respiration. A decrease in CCO complexing and subsequent reduction in CCO activity may evoke marked alterations throughout the mitochondria including loss of nucleic acid (DNA and RNA) as well as protein content, mitochondrial swelling, and disintegration of the outer membrane (60,61).

Since nuclear encoded CCO subunits in the copper-deficient rat heart appear depressed, oligonucleotide probes for subunits II and IV were utilized in Northern hybridization studies (62). It was observed that subunit II expression of mRNA was not altered respective to copper status. Conversely, subunit IV mRNA was either elevated or appeared similar in copper-deficient hearts compared to the controls despite a Western Blot demonstrating a lower level of the encoded protein. Therefore, the reduced protein quantity of subunit
IV appears to be associated with translational or posttranslational events rather than transcriptional events. It has been proposed that the up-regulation of this CCO subunit, and perhaps others, may be the stimulus for an augmentation mitochondrial volume density (62). Thus, the increased mitochondrial-myofibril volume density observed in the copper-deficient rat heart, may in part, be a compensatory mechanism for a lack of the peptides necessary to form CCO.

Additionally, the δ subunit of ATP synthetase has also been observed to be markedly depressed in the copper-deficient rat (56). Like CCO, ATP synthetase is located on the mitochondrial inner membrane and believed to exist adjacent to CCO (60). Also, like CCO, ATP synthetase is an enzyme complex consisting of a many individual nuclear and mitochondrial encoded subunits (63). The bovine heart δ subunit is composed of 146 amino acids (64) and is one of 14 ATP synthetase subunits (63).

Interestingly, despite a reduction in ATP synthetase and CCO quantity, ATP levels do not seem to be effected by copper-deficiency (57,65). A few possible individual or concomitant explanations exist for normal ATP concentrations despite mitochondrial oxidative enzyme reductions. First, electron microscopy has consistently revealed excessive accumulations of glycogen in copper-deficient cardiomyocytes possibly suggesting a greater reliance upon glycolytic mechanisms to meet ATP demands. Second, a possible shift to heavy meromyosin
isoenzyme V₃ which exhibits slower contractility properties thereby decreasing myofibril oxygen demands. Previously, Mercadier et al. (66) observed that isoenzyme V₃ was increased relative to V₁ in the hypertrophic rat heart. However, a relatively greater increase in isoenzyme V₃ may result in gross reductions of cardiac contractility which may be a strong contributing factor to cardiac failure (16). The copper-deficient rat heart may utilize either, neither, or both of these mechanism to conserve cellular ATP concentration. Or it may exclusively or simultaneously utilize other undetermined mechanisms.

The effects of copper deficiency upon the rat heart also seem to be realized interstitially as focal necrosis and subendocardial fibroplasia have been reported (67,68). Copper-deficient rats exhibit a decreased activity of lysyl oxidase which is associated with connective tissue aberrancies. The copper containing lysyl oxidase protein is an extracellular enzyme which is involved in the oxidation of certain lysine and hydroxylysine residues into reactive aldehydes. Such aldehydes can spontaneously form specific cross-links between adjacent collagen chains or collagen and elastin chains (69). Farquharson et al. (70) reported an increased Type III: Type I collagen ratio in copper-deficient rat hearts. Type I and Type III, two of the three most common collagens, form similar fibril structures and can be found in many tissues throughout the mammalian body including: skin,
tendon, bone and muscle (69). It has been reported that
copper-deficient rat electron micrographs display regions of
excessive interstitial collagen deposition (71). This
fibrosis may be a primary result of a copper-deficiency evoked
over-compensatory response to reductions in lysyl oxidase
activity, or, be a secondary effect manifested during cardiac
hypertrophic remodeling. It is possible that a factor of the
copper-deficiency cardiac remodeling process elicits a
stimulus altering the activity of interstitial fibroblasts or,
other collagen metabolism involved cells.

On the other hand, Type IV collagen, forms a two-
dimensional reticulum and is a prominent fraction of all basal
laminas (69). Type IV collagen is composed of three collagen
polypeptides and exists as a triple helical structure
approximately 40 nm in length (69). The helical structure
shows periods of interruption which lend to the flexibility of
the molecule. Typically, four Type IV collagen monomers bind
together at their N-termini to form a characteristic unit.
This unit laterally cross-links with similar collagen units to
form a two-dimensional "chicken wire" apparatus,
characteristic of basal laminae. A common observation of the
copper-deficient rat heart is a disintegration and
discontinuity of an organized pericapillary basal laminae
(9,71). The abnormal basal laminae have shown segments of
thickening as well as stretches of thinning and almost
complete absency. Molecular biology investigation is currently
underway, within our lab, in efforts to better characterize the effects of copper-deficiency upon Type IV collagen and the integrity of the basal laminae. It is possible that the stimulus for metabolic alterations in collagen types is a response to abnormalities in one of the collagen types (IV), or laminin, as a physical response to internal cellular hypertrophic activities. Cardiac myocyte swelling during hypertrophy may distort the molecular design of the connective tissue associated with the plasma membrane and evoke a change in interstitial connective tissue metabolism by signalling fibroblastic activity. Increased fibroblastic presence in copper-deficient rat heart has been suggested previously (71).

Since the componentry of the basal laminae includes not only Type IV collagen, but also, laminin, heparin sulfate proteoglycans and the poorly understood glycoprotein entactin, investigation into the effects of copper deficiency into all of these factors is warranted. Currently, our lab is also involved in the investigation of laminin, in regard to copper-deficiency. Laminin is a cross shaped protein which appears to be the cell membrane anchor site for the basal laminae. In some electron micrographs of copper deficient cardiomyocytes it appears as if the basal laminae is dissociated from the sarcolemma (72), an alteration which may be explained if basal laminae laminin is compromised.

The effects of copper-deficiency upon rat heart ultrastructure and subcellular constituency is apparent
utilizing transmission electron microscopy. Copper-deficiency manifests alterations in subcellular organization including an increased volume density of mitochondria and augmented mitochondria to myofibrillar ratios (8,9). The increased volume density may be a compensatory adaption to a decreased activity of cytochrome c oxidase. Mitochondrial cristae appear in disarray and fragmented (9). Also an increased deposition of collagenous fiber appears to be manifested by the copper-deficiency as mentioned above (9).

Non-invasive examination of the copper-deficient heart is characterized by alterations reflective of the internal changes mentioned above. Electrocardiographic abnormalities include prolonged ST segments, increased PR (PQ) intervals, increased R wave duration and amplitude (6-8) and the presence ventricular and supraventricular beats (7). Utilizing His Bundle electrography, Kopp et al (6) reported increased HV intervals indicative of slow conduction of the His-Purkinje system. Protracted QT duration and augmented QRS amplitude were reported by Medeiros et al (8). Atypical heart rates have been reported (5) although this finding is inconsistent (6).

In regard to investigation of copper-deficiency in the pig, gross anatomical and light microscopic cardiovascular alterations were reported (73,74). Shields and colleagues (73) reported that upon necropsy, the copper-deficient pigs displayed ruptured aortic, cardiac and coronary vasculature and/or papillary muscle rupture. In fact, greater than 50% of
the copper-deficient pigs demonstrated ruptured papillary musculature, the causative effect of copper-deficiency was supported by a lack of similar pathology in a pair fed group.

Copper-deficient pigs also exhibit ultrastructural alterations of the myocardium and atrioventricular valves (75). Other alterations in copper-deficient pigs included enlarged and vacuolated mitochondria and fragmented crista. These pathologies are similar to those reported frequently in regard to copper-deficient rats (8,9).

Selenium

Deficiency of another essential trace element, selenium (Se), has also been shown to have deleterious effects upon the heart and other organs and tissue (10,76). Selenium, atomic number 34, is classified in group VIA of the periodic table of elements. It is considered a metalloid, having properties of both metals and nonmetals. Elemental selenium can be oxidized to the +4 (selenite) or +6 (selenate) oxidation states or reduced to the -2 oxidation state (selenide). The oxidation form of selenium, as found in most supplements of food and feeds, predominate at the higher oxidation state. This is contrary to the selenium metabolites of chief concern within biochemistry, which exists in the reduced state (76).

The utilization of ingested selenium is the net result of complex physiological and metabolic processes that convert ingested selenium to metabolically critical forms vital to
normal function. There are several means of excretion of ingested selenium which decreases the bioavailability for essential Se-proteins. Briefly, in addition to fecal losses of insoluble selenium complexes and poorly digested Se-proteins, selenium may also be lost by methylation and subsequent excretion. Trimethyl selenonium cation is a major portion of urinary selenium loss while volatile dimethyl selenide is expelled by the lungs (76). Selenium is typically metabolized to several compounds, among these are the selenium analogs of the sulfur containing amino acids and therefore to selenium-containing polypeptides and proteins including Se-dependent glutathione peroxidase (76).

The primary nutritional role of selenium is as a constituent of Se-dependent Glutathione Peroxidase (SeGSHpx), an enzyme with antioxidant activities (76). The general activity of this complex metallo-enzyme is as an antioxidant, catalyzing the reaction between a peroxide (ROOH) and glutathione (GSH) resulting in an alcohol (ROH), water and oxidized glutathione (GSSG). GSH is recovered as GSSG and is reduced via glutathione reductase utilizing 2 NADPH (Appendix 1).

The exact catalytic mechanism of SeGSHpx has not fully been determined although models for its mechanism of action have been proposed (77,78). The general consensus is that the selenium bound to the catalytic site occurs in different oxidation states at different steps of the reaction (above and
Appendix 1).

The effects of selenium-deficiency upon animals has been reviewed (76) and includes: degeneration of liver, skeletal, smooth and cardiac muscle, anemia, exudative diathesis, reduced growth and altered collagen metabolism. White Muscle Disease (WMD), also referred to as Nutritional Muscular Dystrophy or "stiff lamb disease", has long been reported in livestock (79). WMD was later associated with areas where the Se content of forage and soils are low (80-82). The WMD preventative effects of Se were demonstrated within the same year by Hogue (83) and Muth (84).

WMD is very common in young growing lambs consuming a diet devoid of both Se (<0.03 ppm Se (dry weight)) and Vitamin E (76). Two clinical patterns have been reported to characterize the occurrence of WMD of these lambs (79,85-87). "Congenital WMD", the first pattern of muscular dystrophy, may result in still borne lambs or lambs which die shortly after birth. Evidence of congestive heart failure is apparent upon post-mortem examination. Varying degrees of subendocardial focal and generally distributed regions of gray-white discolorization, which may involve the subepicardium, are evident. The discoloration, signifying myocardial necrosis, was reported in both ventricles and less frequently in the auricles. Microscopic examination revealed myofibrillar lysis which resulted in noninflammatory coagulative myonecrosis.

"Delayed WMD", the second pattern of muscular dystrophy,
develops within 3-6 weeks or up to 4 months after birth. This pattern is sometimes referred to as "stiff lamb disease" as affected lambs move with a stiff unsteady gait and an arched back. Bilateral focal and diffuse yellow-gray discoloration of skeletal musculature including: diaphragm, intercostels, abductors, longissimus dorsi and triceps femoris are evident. Microscopic examination reveals hyaline degeneration and myofibrillar lysis. Some heart lesions similar to those of the congenital white muscle disease were reported, however, the incidence of cardiomyopathy is low. Abnormal ECGs (including elevated ST segments) and blood pressures were reported by one author (86). Infertility of ewes has also been observed as a characteristic of selenium deficiency (87).

Cheema and Giliani (88) reported gross changes in myocardium in 15 lambs between the postnatal ages of 1 week and 3 months. They reported chalky-white right ventricular endocardium and subendocardial plaques in the interventricular septum and left ventricle. Histological examination also revealed myofibril swelling and fragmentation, myonecrosis, and loss of sarcoplasm and sarcolemmal collapse leading to increased myocardial sarcoplasmic calcium granules.

Nutritional muscular dystrophy and other Se-deficiency related diseases have also been recognized in many other species. In cattle, nutritional muscular dystrophy typically occurs within 1-4 months of age (87) and is manifested by a stiff gait, followed by prostration and death (89). Physical
exertion leads to a rapid onset of signs and death may occur within 1 minute of a period of excitement (89). Myodegeneration is evident at post-mortem examination. This syndrome may be generated by feeding either a diet low in selenium or Vitamin E and exacerbated by feeding increasing amounts of polyunsaturated fatty acids (90).

Dairy cows present a greater incidence of "retained placenta" when fed diets deficient in Se (91-94). This condition involves a failure of the placenta to separate from the maternal crypts in the caruncles post partum. Retained placenta renders the cow more susceptible to uterine infection. Other disease conditions associated with cattle consuming a selenium deficient diet include a greater incidence of cystic ovarian disease (95), "unthriftiness" (reduced growth or body weight loss) (84,96), and anemia associated with Heinz bodies (97).

Selenium deficiency in chickens manifests in three conditions of disease depending upon the specific nutritional circumstances (76). Two of these diseases, exudative diathesis and nutritional muscular dystrophy seem to be preventable with vitamin E supplementation at nutrition levels. The third disease, nutritional pancreatic atrophy, is preventable only with dietary selenium or vitamin E and other antioxidants at greater than nutritional levels (76).

Exudative diathesis is characterized by severe subcutaneous edema, particularly of the abdomen, feet and
ventral neck and wings, anemia, hemorrhaging and hypoproteinemia (98,99). Exudative diathesis in young chicks is produced within 2-4 weeks after hatching by a combined deficiency of dietary Se and vitamin E (76). Noguchi et al. (100) observed significant decreases in hepatic and erythrocytic SeGSHpx activities, the former of which, fell to one third control levels in these chicks.

If the chicks are produced from Se and vitamin E depleted hens and then fed a Se and vitamin E deficient diet after hatching, signs of exudative diathesis will appear as early as 6-12 days (76). Supplements of either Se and vitamin E or both with or without concomitant synthetic antioxidants prevent the disease (101-104).

Nutritional muscular dystrophy in chicks appears to be directly related to vitamin E deficiency and moderately deficient cystine diets (105-110). It has been demonstrated dietary Se is effective at reducing but not fully preventing the nutritional muscular dystrophy in the chick (111,112).

In regard to prevention of nutritional pancreatic atrophy, Thompson and Scott (113,114) reported the necessity of Se. They fed chicks a low Se diet (≈0.01 ppm) but adequate in vitamin E (≈ 100 IU/kg) and found that the chicks required supplemental Se for growth and survival (113). They also observed an impairment of dietary lipid utilization and concomitant decrease in pancreatic enzyme secretion (114). Later studies by Gries and Scott (115), confirmed these
findings by describing degeneration of the acinar pancreas and exocrine function of the pancreas. Pancreatic acinar cells showed vacuolization, loss of zymogen, cytoplasmic shrinkage and dilation of the acinar lumina. Infiltration of fibroblasts and macrophages are observed preceding severe periacinar fibrosis.

Other diseases in the chick which has been reported to decrease in incidence with Se supplementation include encephalomalacia (116), impaired development of immunocompetence (117) and reproductive failure in breeding chickens (118).

Pigs show many disease symptoms related to nutritional intake of Se, vitamin E and synthetic antioxidants and polyunsaturated fats including degeneration of the liver and heart, visceral edema, and abnormal spermatogenesis (76). Pig "hepatosis dietetica", developed by a combined deficiency of vitamin E and Se, is a progressive degeneration of the liver that manifests two clinical patterns. The acute pattern shows acute liver failure that results in sudden death of the pig. Liver lesions include multiple scattered swollen red lobules with hemorrhagic necrosis, and gall bladder wall edema (119-121). Massive necrosis often with hemorrhage is evident upon microscopic evaluation. The second pattern, subacute, is manifested by ascites and jaundice which generally accompanies edema with or without cardiomyopathy. Dispersed lobules of a rough and granular appearance on the surface of the liver are
characteristic of the subacute type.

If growing pigs consume a diet deficient in Se and vitamin E they develop a severe cardiomyopathy characterized by myocardial and epicardial hemorrhages (119,122). The appearance of the afflicted hearts led to the naming "Mulberry Heart Disease" (MHD). MHD may occur with or without hepatosis dietetica and typically exhibits pale streaks on the ventricular myocardium, pulmonary congestion and edema. Vascular and cardiomyocytic lesions are evident upon microscopic examination. Vascular lesions include intramyocardial artery fibrinoid necrosis with fibrin microthrombi in the myocardial capillaries resulting in hemorrhage and edema. The cardiomyocytic lesions include multifocal hyaline necrosis and calcification. Ultrastructural aberrations include myofibrillar lysis, contractile band necrosis, and mitochondrial swelling and mineralization (123,124). Skeletal myopathy is often observed concomitant to either or both hepatosis dietetica and MHD. Pigs with skeletal myopathy resulting from deficiency of Se and vitamin E exhibit a general muscular weakness, unsteady gait, and a spread leg posture ("splay-legged" or "spraddle-legged") (122,125,126).

In regard to humans, Se deficiency received world attention in the late 1970’s when reports of endemic juvenile cardiomyopathy (ie. Keshan disease) and chondrodystrophic disease (Kaschin-Beck disease) were associated with severe selenium-deficiency (11,12). These deleterious effects of
selenium-deficiency upon the human heart were described in discrete regions of China where soil Se contents are very low (11,12). Keshan disease was characterized by cardiomegaly, congestive cardiac failure or cardiogenic shock, arrhythmia and electrocardiogram alterations (13). The diagnosis of Keshan disease was determined on the basis of signs of acute or chronic insufficiency of cardiac function, gallop rhythm, arrhythmia, and electrographic and radiographic anomalies (127,128). It was reported that some subjects demonstrated cardiogenic shock, congestive heart failure and also embolic episodes from cardiac thrombosis (128). Four clinical subtypes of Keshan disease have been established and are discussed in detail elsewhere (127,128).

Briefly, the acute type of Keshan disease shows a rapid onset to apparently healthy children without cardiac history. Symptoms include dizziness, pericardial and substernal discomfort, dyspnea, anorexia, nausea and vomiting. Examination reveals arterial hypotension, pulmonary edema and electrocardiographic aberrancies such as arrhythmia, low QRS voltage, prolonged PQ and QT intervals, AV block, right bundle branch block and inverted T waves. The chronic type of Keshan disease is characterized by chronic congestive heart failure with symptomatology varying with degree of cardiac insufficiency. Symptoms include shortness of breath, cough with hemoptysis, right upper quadrant pain, edema and oliguria. Physical examination reveals cardiac enlargement,
reduced heart sound intensity, gallop rhythm, rales on the base of the lung, hepatomegaly and edema. ECG recordings reveal ventricular or supraventricular premature beats and tachycardia, right bundle branch and A-V block and altered ST segments.

The most prevalent type of Keshan disease is the subacute type which has a greater association with children. It shares similar symptomology as the chronic type but has a more accelerated course. Lastly the latent type is characterized by mild dilatation and associated ECG pattern changes such as right bundle branch block, first or second degree AV block and occasional extraventricular systoles. The subject maintains normal heart function and the disease is usually discovered coincidentally (i.e. autopsy).

In a thorough review of Keshan disease, Ge et al (128) indicated that the primary target organ is the heart although several cases revealed hepatic congestion with excessive fatty deposition and mesenteric lymphadenosis. Gross pathological alterations of the Keshan disease heart includes marked dilatation of primarily the ventricles and secondarily atrial chambers with only moderate hypertrophy of myocardial mass. Interestingly the hearts of the chronic type show a highly expanded spherical shaped heart in contrast to the acute type which may appear normal upon radiographic examination. The subacute type may show some enlargement (128).

Microscopic examination revealed multifocused necrosis
and fibrous replacement of predominantly left ventricular myocardium, although lesions were found throughout (128). Ge et al (128) described two processes of myocardial necrosis: mytocytolysis, represented most often in the subacute type which was suggested to be initiated by mitochondrial degeneration and contractile band necrosis (myofibrillar degeneration predominantly found in the acute type). Chen et al (129) reported marked reduction in succinic dehydrogenase activity in Keshan disease hearts which may support the idea proposed by Ge et al (128) regarding mitochondrial degeneration as an initiating factor. Lastly, Ge et al (128) also recognized the presence of electron-dense particulate inclusions within the cytoplasmic and mitochondrial fractions which were unidentified and suggested to be cardiophilic virus particles.

Since the identification of selenium deficiency as the deleterious factor in Keshan disease other scenarios have been described involving selenium status and cardiac health. Recipients of protracted total parenteral nutrition (TPN) without selenium supplementation are at risk (14-16) and more recently an association between declined selenium status and cardiac complications in patients with Acquired Immunodeficiency Syndrome (AIDS) has been reported (14-20).

As mentioned previously the primary nutritional role of selenium is as a constituent of the antioxidant enzyme complex Se-dependent Glutathione Peroxidase (21). It is assumed that
selenium-deficiency exerts its deleterious effects on the heart by a reduction in protection against oxidative damage (22). The effects of selenium deficiency upon subjects in controlled studies include abnormal electrocardiograms (23), changes in blood pressure (24), decreased basal myocardial contractility (25), and reduced protection against oxygen radical compounds (26).

Iron

By weight iron (Fe) is the Earth’s most abundant element comprising approximately 35% of its total weight. As a transition element iron can exist in several oxidation states varying from Fe$^{6+}$ to Fe$^{2+}$ depending upon the chemical environment. Within the aqueous environment of the human body and food the only stable oxidation states are the ferric (Fe$^{3+}$) and ferrous (Fe$^{2+}$) (130).

The dietary essentiality of iron is attributed to its constituency within heme molecules such as hemoglobin, myoglobin, cytochromes, catalase and peroxidases, non heme molecules such as succinic dehydrogenase and α-glycerophosphate dehydrogenase and as a cofactor for a number of enzymes including aconitase and various deoxygenases (130).

Widely distributed within the food supply of the United States, iron sources include; meat, eggs, vegetables and cereals (especially fortified cereals) (46). Despite the wide distribution of iron in foods, the content of the average
Western diet has been estimated at 6-7 mg/1,000 kcals (131). Historically dietary intake of iron in the United States has been determined inadequate especially in 4 population groups: infants and young children due to food content and rapid growth, adolescents during growth spurts, females during childbearing years due to menstrual losses, and lastly pregnant females due to expanding blood volume and fetal and placental demands (131). Bushnell (132) reported that iron deficiency affects approximately 17-44% of children between 6 weeks and 3 months of age and 25% at 10-15 months of age. The increased Fe RDAs for women (46) is largely attributed to losses and redistribution with menstruation and pregnancy. Bushnell also reported that 10-15% of premenopausal women and 30% of pregnant women may be anemic (132).

Low dietary iron intake along with biological processes of iron loss such as poor absorption, sweat and menstrual loss, bile excretion, desquamation of mucosal and skin cells and urinary excretion can result in the lowering of blood hemoglobin levels due to the involvement of Fe with the erythrocyte heme molecule. Iron deficiency anemia is the most common anemia in the world (132) and in the United States is appears to be the most recognizable nutritional deficiency. Anemia is defined as a reduction in the total concentration of hemoglobin in the blood. Whether the reduction is attributed to a reduction in the hemoglobin content of the erythrocytes or due to a total hematocrit reduction whereby the hemoglobin
is reduced as well, the end result is a decline in the oxygen-carrying capacity of the blood. The type of anemia manifested by iron deficiency is hypochromic microcytic anemia (132).

Chronic anemia in humans (≤ 7 g/dL blood) typically increases cardiac output by augmenting stroke volume and decreases the viscosity of the blood (133). Cardiac output is almost always elevated in severe chronic anemia unless there is an underlying heart disease (134). In the exercising anemic human, developed cardiac output is also typically greater than in the normal human, 1000-1500 ml versus 550-800 ml per 100 ml increase in O₂ consumption per minute (135,136). The increased cardiac output in anemic human subjects is mostly attributed to an exaggerated increase in stroke volume whereby normal subjects respond by increasing heart rate more than stroke volume (135,137).

In view of iron's involvement in many other biological aspects, beyond hemoglobin transport of oxygen, such as electron transport as part of cytochromes, constituency of metallo-enzymes, and as a cofactor for other enzymes, it seems more appropriate to investigate the effects of iron deficiency in a broader sense rather than just the anomalies inflicted by anemia. Finch et al. (138) evaluated work performance on a treadmill in normal and iron-deficient rats in which hemoglobin levels were corrected by catheter exchange transfusion prior to exercise. The iron-deficient animals demonstrated a marked impairment of running ability which was
correctable after 4 days of iron therapy. Biochemical analysis of skeletal muscle showed decreased concentrations of cytochrome and myoglobin pigments and rates of mitochondrial oxidative phosphorylation of pyruvate-malate, succinate and $\alpha$ glycerophosphate substrates. More recent investigation by the same group (139) confirmed the previous observations reporting that iron-deficient rats with lower physical capacity had lower levels of $\alpha$ glycerophosphate oxidase activity. The physiological importance of $\alpha$ glycerophosphate dehydrogenase is in regard to the transfer of extramitochondrial NADH reducing equivalents across the mitochondrial membrane (140). Excessive accumulation of lactate may result in effort to cycle NADH to NAD$^+$ so that glycolysis may continue. Previous efforts reported that $\alpha$ glycerophosphate dehydrogenase isolated from pig brain was a non-heme iron containing complex and the assumption was made that iron's constituency was conserved in the skeletal muscle isoenzyme (141). Several reports have described a decrease in myoglobin concentration in iron-deficient growing rats but not adult rats (139,142,143) and also a decrease in cytochrome c (142,143) and all of the cytochrome complexes (138,143).

The role of anemia in angina pectoris has been attributed to a decreased circulatory oxygen content (144) but it is the intrinsic changes in myocardial metabolic function that may be directly involved in skeletal and cardiac myopathy. Blayney et al (17) reported significant reductions in the activities
of cardiac NADH cytochrome c reductase, succinic cytochrome c reductase, succinic dehydrogenase and NADH ferricyanide oxidoreductase after 7 weeks of iron-restriction and decreased activities of cardiac cytochrome c oxidase and concentrations of cytochromes a3, c1 and b after 14 weeks of iron-restriction. The above enzymes, discounting cytochrome c oxidase, contain nonheme iron and their decreased activities may be attributed to a low availability of iron.

The reduced concentration of cytochrome c reported in skeletal muscle was not complimented by a reduction of cytochrome c in cardiac tissue during iron-deficiency (138,143,145). Finch and colleagues (139) speculated that the difference in cytochrome c concentration was due to a biochemical specificity inherent to the different types of muscle tissue.

Willis et al. (146) examined skeletal muscle mitochondrial non iron-containing and iron containing enzyme activity and proteins of the iron-deficient sedentary and the iron deficient endurance trained rat. Dietary iron deficiency was shown to markedly distort the normal stoichiometric relationship among the constituents of the TCA cycle and the mitochondrial inner membrane.

Iron-deficient rats typically exhibit a reduced body mass versus normal rats (15,17). Iron-deficient rat hearts become hypertrophied and present a heart:body weight ratio (15) and left ventricle:body weight ratio (17) greater than that of the
normal rats. The nature of the hypertrophy is of eccentric character showing enlarged ventricular luminal size with minimal, if any, enlargement of ventricular mass (147). As discussed previously eccentric hypertrophy is often referred to as volume overload hypertrophy and is most definable by the increase in lumen:wall thickness ratio of the left ventricle.

It is without doubt that chronic severe anemia is highly associated with cardiac hypertrophy in humans and experimental animals (148-151) although the intrinsic mechanism for the hypertrophy remains elusive. It has been suggested that norepinephrine is a primary factor in the development of hypertrophy (152,153). Rossi and colleagues (31) investigated the effect of iron-deficiency in the rat upon catecholamine levels and heart morphology. Iron-deficiency was induced by feeding an iron-restricted diet for 30 days post weaning and deficiency was indicated by anemia. The induced cardiac hypertrophy characterized by increased heart wet weight and augmented cell size was highly associated with decreased cardiac norepinephrine levels. The authors speculated that the low cardiac stores of catecholamines in iron-deficiency could mean a larger quantity of neurotransmitter available to bind to cardiomyocyte receptors and stimulate the events leading to protein synthesis. Increases in ventricular chamber radii yields increased tension in the ventricular walls which is proposed as a stimulus for norepinephrine release (35).

In a follow up study by Rossi and colleagues (154),
reserpine, an adrenergic blocking agent was administered daily during iron restriction and compared to a iron-restricted group not receiving reserpine. They reported that the anemia observed in the iron-restricted reserpine administered group was not complimented by cardiac hypertrophy and augmented myocyte cell diameter as observed in the iron-restricted animals without reserpine. In other words, reserpine prevented the development of cardiac hypertrophy in anemic rats. These studies regarding catecholamine metabolism and the induction of cardiac hypertrophy provide fundamental insight into the genesis of iron-deficient cardiac hypertrophy. Also, since cardiac catecholamine concentrations appear to decrease in iron-deficiency, it must then be determined if this is due to a hypersecretion by postganglionic fibers or if the is an earlier abnormality such as an altered tyrosine metabolism to catecholamines.

Electrocardiographic anomalies have been reported in human patients with iron-deficiency anemia (IDA) during an exercise bout (155). ST segment depression was observed in 14 of 55 IDA patients compared to only 1 case in age-sex matched control individuals. Iron-dextran was administered to 12 of the patients showing ST segment depression and the exercise bout was repeated 2-3 days later. ST segment depression was recognized in only one IDA patient receiving iron-dextran therapy. Interestingly, the correction of ST segment depression came prior to elevation of blood hemoglobin levels.
Ultrastructural aberrancies have also been reported in iron-deficient models. Rossi and Carillo (156), examined the cardiac hypertrophy induced by iron-deficiency anemia at the cellular and subcellular level in the rat. Hypertrophy of individual cardiomyocytes were identified together with cellular degradation and interstitial fibrosis. Iron-deficient rats exhibited thickening of Z-lines and proliferation of Z-substance, myofibrillar disarray, marked vacuolization, abnormal appearing intercalated discs, zones of extensive myofilament lysis, and sarcomeres that appeared to have a loss of contractile elements.

Dallman and Goodman (157) demonstrated that iron deficient rats display a greater cardiac mitochondria compartment relative to myofibrils. Elsewhere, similar observations were reported regarding an increased mitochondrial compartment of cardiac myocytes in iron deficiency (158). They reported that markedly enlarged mitochondria seemed to displace and distort the myofibrils.

Johnson et al. (159), investigated the effects of iron-deficiency, in trained and sedentary rats, upon skeletal muscle ultrastructure. In the exercise-trained iron-deficient group, enlarged mitochondria exhibiting sparse cristae were observed. Both the iron-deficient trained and sedentary rats featured lipid droplet accumulation. The iron-deficient trained rats displayed a significantly greater mitochondrial volume than that of the iron-normal trained and iron-deficient
and iron-normal sedentary rats.

Harlan and Williams (160) examined activity-induced adaptations in the tibialis anterior muscle in iron-deficient rabbits. The iron-deficient rabbits consumed a low iron diet (6 μg/g) for 6 months. Muscle contraction of the tibialis anterior was elicited by miniature pulse generators implanted directly below the common peroneal nerve, which innervates the tibialis anterior. Both the control and the iron deficient animals were implanted. The muscle was stimulated into continuous contraction for 10 days. The stimulated iron-deficient skeletal muscle showed an increased mitochondrial fraction characterized by swelling, profound loss of internal membrane structure and surface area, and disruption of the outer membrane.
List of References


CHAPTER III

COMPARATIVE ELECTROCARDIOGRAPHY, GROSS MORPHOMETRY AND ULTRASTRUCTURE OF HEARTS FROM RATS RESTRICTED IN EITHER DIETARY COPPER, IRON, OR SELENIUM.

Abstract

Comparative electrocardiography, cardiac function, gross morphometry and ultrastructural aspects were examined in rats fed a mineral adequate diet or a diet restricted in either dietary copper (<1mg/kg), iron (4mg/kg) or selenium (<0.1mg/kg) for six weeks postweanling. No significant differences were observed in regard to electrocardiograms and gross morphometry of the hearts. The copper-restricted group recorded a significantly lower left ventricular \( \frac{dP}{dt_{\text{max}}} \) in comparison to the mineral adequate diet group. Ultrastructural alterations were readily apparent in the copper and iron restricted cardiac myocytes and subtly apparent in the selenium restricted cardiac myocytes. Both the copper- and iron-restricted rats had comparatively greater mitochondrial volume densities and increased mitochondria: myofibril ratios than the mineral adequate group. All three mineral-restricted groups displayed a lower myofibril volume density compared to
the mineral-adequate group. Vacuolization, myofibril disorganization with Z-line degeneration, and excessive lipid droplet accumulation were apparent in the copper- and iron-restricted groups. Cardiac myofibrillar disarray, disintegration of intercalated discs, and translucent mitochondria were also evident in the iron restricted cardiac myocytes. Selenium restricted mitochondrial membranes appeared disorderly. Subjective evaluation of the pericapillary basal laminae revealed alterations for all three mineral-restricted groups compared to the control group. These results suggest that subcellular alteration are apparent in copper, selenium, and iron restrictions during the development of cardiomegaly and alterations in electrocardiograms.
Introduction

Cardiac pathology has been reported in various species deficient in either dietary copper (1-4), iron (5-7), or selenium (8,9). Although the nutritional roles of these elements differ, some similarities do exist in their general activities. Therefore, common features may be observed in the cardiac pathologies developed when these elements are deficient.

Deficiencies in either copper, selenium, or iron manifest cardiac hypertrophy. Both iron (6,7) and selenium (9) deficiencies have been reported to evoke an eccentric pattern of hypertrophy, contrary to the concentric hypertrophy characteristic of copper deficiency (1).

Electrocardiographic abnormalities have also been reported in all three models of mineral deficiency. Copper deficiency can elicit elongation of QT interval (10), augmented R wave amplitude and duration (3,11), prolonged QRS complexes (10), and slurring of the isoelectric baseline (2,3). Iron deficiency has been reported to cause ST segment depression in human subjects during exercise (12). Electrocardiographic aberrations have also been reported in selenium deficient humans (9) and animals (13,14).

Subcellular alterations are often the basis for the cardiac pathology recognized upon gross invasive or noninvasive examination. Electron micrographs from copper deficient rats (2,3,10) and pigs (4) have shown shifts in cardiac myocyte
volume densities, with a greater mitochondrial fraction, disfigured mitochondrial inner membranes, and increased collagen deposition and alterations of basal laminae in the interstices. Iron deficient rats exhibit alterations of Z-line structure, myofibrillar disarray, marked vacuolization, abnormal intercalated discs, and myofilament lysis (15). Lipid droplet accumulation has been noted in both copper (1,3) and iron deficient (16) rat micrographs. Subcellular alterations have also been reported in selenium deficiency (3,9). Subtle alterations in myocardial volume densities have been reported in rats fed a selenium deficient diet for 8 weeks post weaning (3), while a more protracted selenium restriction is manifested by mitochondrial degeneration and contractile band necrosis (9).

The purpose of this study was to comparatively examine the dietary restrictions of either copper, iron, or selenium as they affect the heart and its function. This study will yield insight to similarities and differences between the three nutrition related cardiomyopathies in regard to electrocardiography, developed ventricular pressures, gross morphometry and subcellular characteristics.
Materials and Methods

**Animal diets.** Weanling male Long-Evans rats (n = 28) were purchased from Harlan Sprague Dawley (Indianapolis, IN). Upon arrival the animals were weighed and randomly assigned to four diet treatment groups.

The rats were fed a basal diet that complies with the recommendations of the American Institute of Nutrition (17), consisting of (by weight) 50% sucrose, 20% casein, 15% cornstarch, and 5% corn oil as energy sources. One group (n=6) received copper (cupric carbonate) at 6 mg/kg diet, selenium (selenium selenite) at 0.2 mg/kg diet and iron (ferric chloride) at 40 mg/kg diet (mineral-adequate (CON)). The other three treatment groups received diets in which either copper was omitted (copper-restricted (Cu-R) (< 1mg/kg) (n=8), selenium was omitted (selenium restricted (Se-R) (<0.1 mg/kg) (n=6) or iron was reduced to 4 mg/kg (iron restricted (Fe-R)) (n=8). Mineral mixtures of these specifications were purchased prepared from U.S. Biochemical Corporation (Cleveland, OH). Actual copper, selenium, and iron content were determined by flame atomic spectrophotometry. Deionized-distilled water was allowed without restriction throughout the duration of the study.

Rats were housed singly in stainless steel cages in a controlled environment with a 12 hour light:dark cycle at constant room temperature and humidity. All animals were fed their respective diets for 6 weeks and body weights were
obtained weekly. This protocol was approved by The Ohio State University Institutional Animal Welfare Committee.

**Electrocardiograms.** At the closure of week 6, six rats from the Cu-R and Fe-R groups and four rats from the CON and Se-R groups were fasted overnight and anesthetized with ketamine (85 mg/kg body weight) and xylazine (15 mg/kg body weight) injected intraperitoneally. Electrocardiographic leads were established by subcutaneous needle electrodes. Leads I, aVF, and V3 were recorded at 100 mm/sec paper speed on a photographic oscillograph, with a frequency response flat to over 1000 Hz. Leads I and aVF were utilized because they permit representation of projections of cardiac vectors on the two axes (I = X, aVF = Y) constituting the frontal plane. Precordial lead V3 was utilized because it permits a greater proximity to the left ventricular free wall, and therefore would be sensitive to alterations in ventricular depolarization and repolarization. The following ECG parameters were analyzed from leads aVF and V3: PQ, QRS, and QT intervals; amplitude of the R wave; and peak to peak value of R to S (R:S ratio only in V3). Heart rate was determined from chronological assessment of P-waves in lead I. The paper speed of 100 mm/sec allowed for the resolving of intervals to at least 10 msec. Subjective evaluation of the ECG was performed noting: changes in the contour of the QRS complex and/or QT interval in leads aVF and V3, occurrence of high frequency notching or slurring to QRS, and alterations in
polarity of component deflections. The R:S ratio in lead V3 was evaluated because its ratio changes in different directions with right or left ventricular hypertrophy in dogs and cats.

**Hemodynamic evaluation.** Immediately following the ECG, a short-beveled, 21-gauge hypodermic needle, attached directly to a pressure transducer, was thrust through the left thoracic wall entering the left ventricle. Pulsatile pressures and their derivatives were recorded using a modification of the method of Saragoca and Tarazi (18). Maximal rates of left ventricular luminal pressure rise and fall, LV \(+/dP/dt_{max}\), and total developed left ventricular pressure, \(dP_{LV}\), were recorded. The frequency response of the overall recording system is flat to over 150 Hz. If a tracing was not obtained after two attempts the rat was removed as repeated attempts may compromise cardiac integrity and influence the tracing. Therefore, data for LV \(dP/dt_{max}\) \(dP_{LV}\) measurements may represent fewer than the maximal number of rats for each group.

**Heart Dimensions.** Immediately following the cardiac tests and while still fully anesthetized, the thoracic cavities were opened by midline incision. Blood was drawn by a cardiac puncture and placed in heparinized vials for biochemical tests, and then the rats were sacrificed by severing the aorta.

The hearts were removed, rinsed in 0.9% saline, blotted dry, weighed and then transferred to 10% formalin for later
morphometrical analysis. Median coronal sections were cut and gross morphometric measurements were obtained by calipers utilizing procedure described previously by Jenkins & Medeiros (19). Measurements included width of the left ventricular and right ventricular free walls and the intraventricular septum, apical dimension and left ventricular major length and left ventricular lesser diameter.

Tissue preparation for transmission electron microscopy analysis. The remaining 2 animals from the four treatment groups were utilized the ensuing day for electron microscopy utilizing a slightly modified procedure as described by Medeiros et al. (20). The modification employs the utilization of Sorenson's PBS buffer rather than sodium cacodylate containing arsenic. Briefly, the hearts were perfused in 2.0% glutaraldehyde in 0.1 mmol/mL Sorenson's PBS in .1mmol/mL sucrose (Electron Microscopy Sciences, Fort Washington, PA). Hearts immersed in the glutaraldehyde solution were first cut (Electron Microscopy razor blades, Electron Microscopy Sciences, Fort Washington, PA) on a transverse plane and then cut transversely again producing a "cardiac donut". Left ventricular free wall tissue samples were cut (< 1 mm) tangentially to the outer wall producing myofibers in a longitudinal plane. Preparation of the tissue samples for TEM followed the procedure described previously (20) utilizing a series of acetone dehydrations and Spurr resin embedment. Embedment molds were then allowed to
polymerized in a vacuum oven at 60°C overnight. The resin-tissue blocks were then thick sectioned to ensure appropriate vantage of longitudinal myofibers and then thin sectioned with a Diatome diamond knife (Electron Microscopy Sciences, Fort Washington, PA). Thin sections (± 90 nm) were caught on mesh Cu grids (75 x 300) and examined by TEM (Philips EM 301B, Eindhoven, The Netherlands), at 60 kV, and the negatives developed and printed to desired magnifications.

**Morphometrical analysis of electron microscopy.** Electron micrograph prints were analyzed as described elsewhere by Medeiros et al. (20). Volume densities (μm³/μm³) of mitochondria, myofibrils and "other" intracellular material were determined using print enlargements of 18,800 X that followed a point system previously described by Weibel (21) and Steer (22). "Other" intracellular material is that which is not clearly defined as either mitochondria or myofibril. Volume density average scores for each diet group were calculated from a mean score of three micrographs for both rats in a diet group. The data are displayed as comparative averages due to a sample size n = 2 per treatment group.

**Liver selenium-dependent glutathione peroxidase.** Livers from all rats were removed, rinsed in deionized-distilled water, blotted dry, weighed and frozen until later enzyme analysis.

Liver glutathione peroxidase activity was determined by a coupled assay method originally described by Paglia and
Valentine (23) and later modified by Levander et al. (24). Enzyme activity was expressed as Units, where 1 Unit = 1 μmole of NADPH oxidized/minute. SeGSHpx activity was expressed as Units/mg protein (U/mg).

**Liver Cu and Fe and diet Cu, Fe, and Se determination.** Liver and diet samples were wet digested in 70% nitric acid as previously described (25). Liver Cu and Fe were determined utilizing a Perkin-Elmer flame atomic absorption spectrophotometer (Norwalk, CT). Reference bovine liver purchased from the National Institute of Standards and Technology (Gaithersburg, MD) was used to verify the accuracy of the results. After wet ash digestion diet samples were sent to Stilson Laboratories, Inc (Columbus, OH) to measure Se content by similar methodology. Diet content of Cu, Fe and Se was determined prior to the onset of the study.

**Hematocrit and Hemoglobin.** Heparinized blood was transferred to microhematocrit tubes and centrifuged in a microcapillary centrifuge. Hematocrit readings were measured to the nearest 0.005. Hemoglobin was determined by dilution of blood sample in Drabkin's reagent (Sigma Chemical Inc, St. Louis, MO.) whereby hemoglobin, methemoglobin, and carboxyhemoglobin were converted to cyanmethemoglobin which was measured spectrophotometrically at 540 nm.

**Statistical Analysis.** With respect to the limited sample sizes, data were analyzed by both parametric and nonparametric statistical tests. Parametric ANOVA and Kruskal-Wallis
Nonparametric ANOVA tests were performed to determine if a significant difference existed in the variations of sample means and medians, respectively. Post ANOVA tests were performed when a difference was determined utilizing the above ANOVA procedures. The Tukey-Kramer Multiple Comparison Post-ANOVA Test was utilized to determine which means differed for the parametric analysis while the Dunn Multiple Comparison Post-ANOVA Test was employed for nonparametric median difference determination. The $\alpha$ level was set at 0.05.

The text includes results and discussion in regard to parametric tests only. Data in tables indicate significant differences determined by both parametric and nonparametric tests.
Results

All animals completed the six week diet treatment period. The Fe-R animal body weights were significantly lower than the CON, Cu-R and Se-R group body weights after 6 weeks of treatment (p<0.05) (Table 1 and Figure 1). Both Cu-R and Se-R groups also showed a decelerated body weight gain in comparison to the CON animals although those differences were not significant (Figure 1).

The significant decelerated body weight gain in the Fe-R group was accompanied by a lower absolute heart weight in comparison to the CON, Cu-R and Se-R after six weeks of diet treatment (Table 1, p<0.05). However, heart weight:body weight ratios were not different (p>0.05) between any of the diet groups.

Selenium-restriction yielded a significant reduction (p<0.05) in liver SeGSHpx activity for the Se-R group in comparison to the other groups (Table 1). Concurrently, liver Cu and Fe concentrations were markedly effected by dietary Cu and Fe restrictions. The Fe-R group liver Fe was significantly lower than the other three groups (Table 1, p<0.05). Contrarily, Cu-R group liver Fe was greater than the other three diet groups (Table 1, p<0.05). Liver Cu was significantly lower in the Cu-R group in comparison to the other three groups (Table 1, p<0.05).

Hemoglobin levels were not significantly different between any of the diet treatment groups after 6 weeks while
### TABLE 1

**Body and Heart Weight, Heart Weight:Body Weight, Liver Cu, Fe, SeGSHPx, Hemoglobin and Hematocrit of Rats Fed CON, Cu-R, Fe-R and Se-R Diets**

<table>
<thead>
<tr>
<th>Indices</th>
<th>CON (n=6)</th>
<th>Cu-R (n=8)</th>
<th>Fe-R (n=8)</th>
<th>Se-R (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Wt.</strong> (grams)</td>
<td>318.3 ± 18.1&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>277 ± 41.5&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>232.8 ± 23.1&lt;sup&gt;Bb&lt;/sup&gt;</td>
<td>296.5 ± 26.5&lt;sup&gt;Aa&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Heart Wt.</strong> (grams)</td>
<td>1.22 ± 0.07&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>1.25 ± 0.15&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>0.96 ± 0.09&lt;sup&gt;Bb&lt;/sup&gt;</td>
<td>1.20 ± 0.06&lt;sup&gt;Aab&lt;/sup&gt;</td>
</tr>
<tr>
<td>**Heart Wt:**Body Wt. (x 10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>3.8 ± 0.4</td>
<td>4.4 ± 0.3</td>
<td>4.1 ± 0.1</td>
<td>4.1 ± 0.4</td>
</tr>
<tr>
<td><strong>SeGSHPx</strong> (U/mg)</td>
<td>1.18 ± 0.24&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>0.91 ± 0.21&lt;sup&gt;Aab&lt;/sup&gt;</td>
<td>1.11 ± 0.02&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>0.08 ± 0.01&lt;sup&gt;Bb&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Liver Fe</strong> (ppm)</td>
<td>140 ± 41.9&lt;sup&gt;Aab&lt;/sup&gt;</td>
<td>265 ± 39.6&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>82.5 ± 22.5&lt;sup&gt;Cb&lt;/sup&gt;</td>
<td>146.7 ± 27.3&lt;sup&gt;Aab&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Liver Cu</strong> (ppm)</td>
<td>6.9 ± 0.2&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>0.6 ± 0.3&lt;sup&gt;Bb&lt;/sup&gt;</td>
<td>6.8 ± 0.4&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>6.8 ± 0.4&lt;sup&gt;Aa&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Hemoglobin</strong> (g/dL)</td>
<td>11.2 ± 2.8</td>
<td>10.1 ± 1.3</td>
<td>9.1 ± 2.16</td>
<td>12.1 ± 2.4</td>
</tr>
<tr>
<td><strong>Hematocrit</strong> (%)</td>
<td>45.9 ± 4.6&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>43.9 ± 1.7&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>39.4 ± 6.1&lt;sup&gt;B&lt;/sup&gt;</td>
<td>46.4 ± 4.6&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values expressed as means ± SD. Values with different superscripts are significantly different (p<0.05) where upper case and lower case letters signify differences determined by parametric and nonparametric analysis, respectively.
Figure 1

Weekly Body Weights

Weekly body weight change for the CON, Cu-R, Fe-R, and Se-R groups. The Fe-R group body weights were lower than the other three groups after 6 weeks of dietary restriction \((p<0.05)\).
Fe-R body weights were lower than the other groups (p<0.05)

Figure 1.
the Fe-R hematocrit was significantly lower than the Se-R hematocrit levels (Table 1, p<0.05).

LV -dP/dt max, and dP_LV measurements did not differ between groups (Table 2). However, the Cu-R group LV +dP/dt max was significantly lower than the CON group (p<0.05) (Table 2 and Figure 2).

Electrocardiographic measurements obtained from leads aVF and V3 did not reveal any significant findings in either lead (Table 3 and Figure 3).

Gross morphometrical assessment did not reveal any absolute significant differences between groups in regard to heart structure (Table 4). Coronal heart sections are shown on Plate I.

Ultrastructural examination (Plate II-VI) revealed greater mitochondrial volume densities for the Fe-R and Cu-R groups in comparison to the CON and Se-R groups (Figure 4). Myofibril volume densities were reduced in Cu-R, Fe-R, and Se-R groups in comparison to the CON group. Ultrastructural alterations for the Fe-R micrographs included regions of: excessive mitochondrial volume, sparse cristae of mitochondria yielding a translucent appearance, myofibril disarray with myofibrils coursing in different directions, increase lipid droplet accumulation, myofibril disorganization, thickening and disruption of Z-lines, regions of disintegrating intercalated discs, disorganization of basal laminae and focal interstitial fibrosis, and excessive vacuolization. Cu-R
micrographs exhibited increased mitochondrial volume densities and mitochondrial:myofibril ratios, excessive lipid droplet accumulation, myocardial fibrosis, and interstitial basal laminae distortion and excessive collagen deposition. Se-R micrographs showed increased spacing between myofibrils and perimitochondrial regions which augmented the "other" fraction of the volume density analysis. Se-R electron micrographs also showed disorderly mitochondrial membranes and interstitial basal laminae disruption and thickening.
TABLE 2

*Developed Ventricular Pressure Measurements of Rats Fed CON, Cu-R, Fe-R and Se-R Diets*¹

<table>
<thead>
<tr>
<th></th>
<th>CON (n=4)</th>
<th>Cu-R (n=5)</th>
<th>Fe-R (n=5)</th>
<th>Se-R (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LV +dP/dtmax</strong>²</td>
<td>13433.3 ± 2205⁹</td>
<td>9733 ± 1450⁹</td>
<td>10370 ± 1705⁹AB</td>
<td>12500 ± 2200⁹AB</td>
</tr>
<tr>
<td>(mm Hg/sec)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LV -dP/dtmax</strong>³</td>
<td>9333 ± 923</td>
<td>6983 ± 2479</td>
<td>7740 ± 2265</td>
<td>9100 ± 1465</td>
</tr>
<tr>
<td>(mm Hg/sec)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>dP_LV</strong>⁴,</td>
<td>133.3 ± 20.6</td>
<td>125.4 ± 15.8</td>
<td>143.9 ± 42.4</td>
<td>135.2 ± 13.7</td>
</tr>
<tr>
<td>(mm Hg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹Values are expressed as means ± SD. Values with different superscripts are significantly different by parametric analysis (p<0.05).
²dP/dtmax = maximum rate of pressure development.
³dP_LV = total left ventricular pressure development.
Select LV Pressure Curves and $dP/dt$s

Select Electrocardiograms and LV pressure curves $dP/dts$ from all groups. There were no differences in $dP_{LV}$ between the groups, however, Cu-R LV $+dP/dt_{max}$ was lower than the other groups ($p<0.05$).
Figure 2.
<table>
<thead>
<tr>
<th></th>
<th>CON (n=4)</th>
<th>Cu-R (n=6)</th>
<th>Fe-R (n=6)</th>
<th>Se-R (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heart Rate</strong></td>
<td>296.9 ± 34.6</td>
<td>306.1 ± 26.3</td>
<td>286.6 ± 43.5</td>
<td>319.3 ± 28</td>
</tr>
<tr>
<td><strong>ECG components (lead aVF):</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PQ interval, (mS)</td>
<td>45.7 ± 4.0</td>
<td>45.0 ± 4.9</td>
<td>43.0 ± 4.6</td>
<td>46.6 ± 4.5</td>
</tr>
<tr>
<td>QRS duration, (mS)</td>
<td>17.8 ± 1.8</td>
<td>19.3 ± 2.3</td>
<td>17.6 ± 0.8</td>
<td>17.4 ± 2.9</td>
</tr>
<tr>
<td>QT duration, (mS)</td>
<td>86.7 ± 8.7</td>
<td>100.37 ± 7.6</td>
<td>96.9 ± 7.8</td>
<td>99.8 ± 12.8</td>
</tr>
<tr>
<td>R wave, (mV)</td>
<td>0.25 ± 0.15</td>
<td>0.32 ± 0.20</td>
<td>0.31 ± 0.25</td>
<td>0.24 ± 0.04</td>
</tr>
<tr>
<td><strong>ECG components (lead V3):</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PQ interval, (mS)</td>
<td>41.9 ± 3.2</td>
<td>44.2 ± 3.7</td>
<td>43.5 ± 2.6</td>
<td>44.5 ± 3.0</td>
</tr>
<tr>
<td>QRS duration, (mS)</td>
<td>17.3 ± 1.8</td>
<td>17.5 ± 1.7</td>
<td>17.7 ± 0.8</td>
<td>18.3 ± 3.8</td>
</tr>
<tr>
<td>QT duration, (mS)</td>
<td>79.5 ± 14.2</td>
<td>93.0 ± 10.8</td>
<td>88.2 ± 22.5</td>
<td>77.3 ± 6.9</td>
</tr>
<tr>
<td>R wave, (mV)</td>
<td>0.53 ± 0.10</td>
<td>0.28 ± 0.18</td>
<td>0.33 ± 0.18</td>
<td>0.30 ± 0.14</td>
</tr>
<tr>
<td>R:S</td>
<td>3.2 ± 1.7</td>
<td>1.4 ± 0.8</td>
<td>1.1 ± 0.9</td>
<td>1.1 ± 1.0</td>
</tr>
</tbody>
</table>

*Values are expressed as means ± SD. No significant differences were determined.*
Select Electrocardiographic tracings

Select ECG tracings from all groups. No significant differences were determined between groups for any of the parameters.
Figure 3.
TABLE 4


<table>
<thead>
<tr>
<th>Measure (mm)</th>
<th>CON (n=4)</th>
<th>Cu-R (n=6)</th>
<th>Fe-R (n=6)</th>
<th>Se-R (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS</td>
<td>3.71 ± 0.23</td>
<td>3.61 ± 0.29</td>
<td>3.70 ± 0.5</td>
<td>4.10 ± 0.47</td>
</tr>
<tr>
<td>LVFW</td>
<td>3.47 ± 0.24</td>
<td>3.86 ± 0.41</td>
<td>3.64 ± 0.27</td>
<td>3.60 ± 0.20</td>
</tr>
<tr>
<td>RVFW</td>
<td>1.33 ± 0.28</td>
<td>1.52 ± 0.27</td>
<td>1.36 ± 0.27</td>
<td>1.30 ± 0.22</td>
</tr>
<tr>
<td>LVML</td>
<td>10.92 ± 0.82</td>
<td>10.54 ± 0.65</td>
<td>10.20 ± 0.44</td>
<td>9.79 ± 1.18</td>
</tr>
<tr>
<td>LVLD</td>
<td>2.71 ± 0.28</td>
<td>2.48 ± 0.37</td>
<td>2.15 ± 0.44</td>
<td>2.04 ± 0.44</td>
</tr>
<tr>
<td>AD</td>
<td>2.15 ± 0.31</td>
<td>2.40 ± 0.44</td>
<td>1.92 ± 0.71</td>
<td>2.73 ± 0.43</td>
</tr>
<tr>
<td>LVFW:LVLD</td>
<td>1.30 ± 0.23</td>
<td>1.58 ± 0.20</td>
<td>1.76 ± 0.44</td>
<td>1.78 ± 0.11</td>
</tr>
</tbody>
</table>

Values expressed as means ± SD. No significant differences were determined.

IVS = interventricular septum
LVFW = left ventricular free wall
RVFW = right ventricular free wall
AD = apical dimension
LVML = left ventricular major length
LVLD = left ventricular lesser diameter
Plate I

Coronal Sections of Hearts

Coronal sections of hearts of CON (top row), Cu-R (second row), Fe-R (third row), and Se-R (bottom row) diet groups. Morphometric measurements did not determine differences between the groups.
Plate II

Electron Micrographs: Volume Density

Transmission Electron Micrographs displaying volume densities and cardiomyocyte organization of the CON (A), Cu-R (B), Fe-R (C & D), and Se-R (E & F) diet fed groups. Cu-R, Fe-R, and Se-R groups showed reductions in myofibrils, while Cu-R and Fe-R displayed increased mitochondrial compartments. Excessive lipid droplet inclusions are obvious in the Cu-R micrograph. Fe-R micrographs present translucent mitochondria and thin myofibrils. M = mitochondria; my = myofibril; T = T-tubule; L = lipid droplet. Bar = 1 µm.
Plate III

Electron Micrographs: Myofibril Disruption and Myofibril Disarray

Transmission Electron Micrographs displaying Fe-R myofibril disruption (A) and myofibril disarray (A). Note in (B) the large headed arrow pointing to a Z-line. M = mitochondria; my = myofibril; T = T-tubule; L = lipid droplet. Bar = 1 μm.
Plate IV

Electron Micrographs: Mitochondria

Transmission Electron Micrographs displaying mitochondrial structure of CON (A), Cu-R (B), Fe-R (C), and Se-R (D). Fe-R mitochondria appeared translucent due to cristae scarcity and disruption and vacuolization. The Se-R mitochondria appeared to have a disorganized bi-membrane system. Note the thin arrow in Cu-R and Fe-R indicating glycogen inclusion. M = mitochondria; my = myofibril; T = T-tubule; V = vacuole. Bar = 1 µm.

92
Plate IV (continued)
Plate V

Electron Micrographs: Basal Laminae

Transmission Electron Micrographs displaying the perivascular space and myocyte and endothelial basal laminae of CON (A), Cu-R (B & C), Fe-R (D), and Se-R (E). Subjective analysis determined abnormalities in the organization of the basal laminae (thin arrows). M = mitochondria; my = myofibril; T = T-tubule; L = Lipid droplet; i = interstitial space; E = endothelial cell; c = capillary lumen; e = erythrocyte. Bar = 1 μm.
Plate VI

Electron Micrographs: Perivascular Space

Transmission Electron Micrographs displaying the perivascular space of CON (A), Cu-R (B & C) and Fe-R (D). Cu-R micrographs present excessive collagen deposition, myocyte fibrosis and the presence of Mast cells and Fibroblasts. The Fe-R micrograph displays focal regions of excessive collagen fibrosis. Note the short arrows points to collagen or related protein fibers. M = mitochondria; my = myofibril; T = T-tubule; Lipid droplet; i = interstitial space; E = endothelial cell; c = capillary lumen; n = nucleus; Ma = Mast cell; F = fibroblast. Bar = 1 μm.
Figure 4
Subcellular Volume Densities

Comparative average of subcellular volume densities for all groups. All three mineral restricted groups displayed marked reductions in myofibril volume densities (VMYO) while Cu-R and Fe-R showed dramatic increased mitochondrial volume (VMITO) and mitochondrial volume:myofibril volume density ratios (VMITO:VMYO).
Figure 4.

Subcellular Volume Densities

CON Cu-R Fe-R Se-R

Diet Treatment Group

VMITO VMYO VOTH □ VMITO:VMYO

Comparative averages
Discussion

The effect of diets deficient in either copper (1-4,10,11), iron (5-7,15,16), or selenium (8,9,13,14), have received considerable research interest. Deficiencies in all three essential trace metals are manifest by pronounced cardiomyopathies. The similarities and differences in the cardiomyopathies associated with consuming a diet restricted in their respective trace metal for 6 weeks postweaning were investigated.

Animals consuming diets with extremely low levels of either copper (3,26,27), iron (6,7,28,29), or selenium (30-35) have demonstrated decelerated body weight gain or body weight loss. In the present study both the Cu-R and Se-R group body weights were lower, although not significantly, than the CON group after 6 weeks. However, the Fe-R group developed a mean body weight significantly lower than the CON and the Cu-R and Se-R groups. Deficiencies in both copper (1,3,26,27) and iron (6,7,28,29) result in reductions in hematocrit and hemoglobin, yielding anemia. Although a trend of reduced hematocrit and hemoglobin in comparison to the CON group were presently observed in the Cu-R and Fe-R groups, they were not statistically significant. However, the hematocrit of the Fe-R group was determined lower than the Se-R group.

Gross cardiac hypertrophy has been reported with all three metal deficiencies. Copper deficiency has shown a concentric pattern of hypertrophy as demonstrated by greater
absolute heart weights, heart weight:body weight ratios and ventricular, interventricular and apical wall thicknesses (3,4,26). The eccentric pattern of hypertrophy associated with iron deficiency is characterized by enlarged myocytes (28), greater specific heart weight (g/100 g of body weight) (6), and greater ventricle:body weight ratios (7). The cardiomegaly reported in association with selenium deficiency is more affiliated with the eccentric pattern of hypertrophy as the dilatation of the ventricular chambers has been reported to account for most of the cardiac enlargement in humans (36). Heart weight:body weights, and measurements of ventricular dimensions were not significantly different in the any of the mineral restricted groups after 6 weeks.

Electrocardiographic aberrations were not determined between groups in the present study utilizing leads aVF and V3, although a trend towards QT protraction was evident in all three mineral-restricted groups. Electrocardiographic aberrations have been a consistent finding of copper deficient rats (1-3,10,27). Abnormalities include: ST segment alterations, protracted PR intervals, and R wave duration and amplitude (1,2,37). In iron deficiency models, significant ST segment depression have been reported (12,38). Electrocardiographic aberrations have also been associated with selenium deficiency (9,13,14). Godwin (13) described a pattern of developed electrocardiographic abnormalities beginning with P-waves on preceding T-waves (P/T). As the
animal progresses toward death bradycardia, increased T-wave voltages and ST elevation are observed.

The Cu-R group did exhibit a significantly lower LV $+\frac{dP}{dt_{\text{max}}}$ than the CON group. The LV $+\frac{dP}{dt_{\text{max}}}$ measurement utilized in the present study, is an indicator of left ventricular systolic function. Left ventricular systolic function is primarily affected by preload, in accordance with the Frank-Starling law of the heart, and myocardial contractility. As heart rates were similar and previous work has observed normal levels of ATP in copper-restricted rats (39), a predominant influencing factor would seem to be a reduction in sarcomere contractility. Currently our lab is investigating forms of isomyosin types present in copper-restricted rat hearts to determine if a there is a shift from the normally present $V_1$ isoenzyme to a more slowly contractile $V_3$ isoenzyme in the copper-deficient rat. Results from two unpublished studies did demonstrate a significant ($p<0.05$) shift to $V_3$ in copper-deficient rats. Also the sarcomere disorganization typically recognized in copper-deficient animal micrographs could hinder proper cross-bridging and contractile efforts.

Besides myocardial hypertrophy, cardiomyopathy may result in other changes in the appearance of an inflicted heart. In selenium deficiency, two pronounced visual alterations have been reported, both independently and concurrently, by several authors. The cardiomyopathy in growing pigs fed diets
deficient in both selenium and vitamin E is manifested by pale, chalky streaks ("White Muscle Disease" (WMD)), and/or widespread hemorrhage yielding discoloration ("Mulberry Heart Disease"(MHD)) (39-41). WMD has been reported in growing lambs feeding in regions poor forage and soil content of selenium (42,43). The WMD in lambs (also known as "stiff lamb disease" or "nutritional muscular dystrophy") is similar to the pig model. Post mortem examination of WMD inflicted lamb hearts typically reveals focal or diffuse discolorization involving the subepicardium and extending deep into the myocardium (44). The discolorization signifies regions of myonecrosis and calcium infiltration. In the current study, pale discoloration of ventricular epicardium was observed in one of the Se-R rats upon sacrifice.

External or gross characteristics of cardiomyopathies are often a cumulative expression of alterations at the cellular and extracellular levels. The cardiomyopathies associated with deficiencies of either dietary copper, iron, or selenium are without exception to this concept. Copper-deficiency is manifested at the ultrastructural level by augmentations of mitochondrial volume density and mitochondria:myofibril volume density ratios (3,4,5,45). The decreased myofibril volume densities observed (10,26) is more likely attributable to a rapidly increasing mitochondrial and "other" compartments during hypertrophic remodeling versus marked myofibrillar degradation. Disorganization of myofibrils (4), and
intercalated discs (45), and increased intracellular inclusions of glycogen granules and lipid droplets are also recognizable in copper-deficient cardiomyocytes (4). Alterations of perivascular compartments include increased collagen deposition and disruption of the basal laminae (45). Electron micrographs from the present study presented marked augmentation of mitochondrial volume density and mitochondria:myofibril volume densities ratio. Some evidence of mitochondrial enlargement was noticed along with increased accumulation of lipid droplet inclusions, both of which appeared to disrupt myofibril organizational patterns. Focal fibrosis was apparent within the myocardium. Disruption of the basal laminae and excessive collagen deposition marred the perivascular space.

Cardiac myocytes from the iron-deficient animals also show alterations in volume densities, with an increase in the mitochondrial compartment (5). Rossi and Carillo (15) investigated the hypertrophied myocardium of iron-deficient anemic rats. They reported misalignments, irregularities and thickening of Z-lines, and regions of ultrastructural alterations of myofibrils, mitochondria and sarcoplasmic reticulum. Some mitochondria exhibited "clearing of the matrix" and disruption of cristae. Focal widening of the interstitial space was observed due to cellular infiltration by mononuclear cells and fibroblasts with collagen fibers. These microscopic findings are supported by our observations
as Fe-R rat micrographs displayed augmentation of mitochondrial volume density and an increased mitochondria: myofibril volume density ratio, similar to the Cu-R micrographs. The Fe-R micrographs also presented increased lipid droplet inclusions and a disorganization of the basal laminae. Large regions of mitochondrial compartment expansion were also obvious. The mitochondria appeared enlarged and translucent due to sparse and fragmented cristae. Myofibrils were disorganized with Z-line disruption. Focal regions of myofibrillar disarray were also observed along with disorganization of intercalated discs.

In regard to selenium-deficiency, Cheema and Giliani (46) observed cardiac myocyte necrosis and a heavy deposition of calcium in lambs with WMD. Microscopic examination of selenium- and vitamin E-deficient pigs revealed ventricular myocyte lesions (39,47). Mitochondrial swelling and mineralization, myofibrillar lysis and contractile band necrosis were reported. The mitochondrial membrane structure in our Se-R rat micrographs seemed disorderly. A lack of membrane structural integrity would certainly allow for the mitochondrial changes cited above.

Previously (3), in rats fed a selenium-restricted diet for 8 weeks, increased myocyte volume densities of "other" material were observed, which occupied a significant portion of cytoplasm, possibly suggestive of edema. Also, some evidence of mitochondrial swelling was apparent. Presently,
the observed increase in "other" volume density is comprised of everything that is not mitochondria or myofibril. This would include cytoplasmic fluid, intercalated discs, nuclei, T-tubule-sarcoplasmic reticulum networks and cellular inclusions such as lipid droplets and glycogen granules. It appeared that there was increased separation between some myofibril bundles, mitochondria, and between mitochondria and myofibrils. Interestingly, the effects of selenium restriction may be realized earlier in perivascular spaces as the basal laminae of the Se-R group micrographs appeared disarranged. It has been reported previously that sheep afflicted with WMD show increased activities of collagenase (48).

In conclusion, the results of the present study suggest that the cardiomyopathies associated with deficiencies of either copper, iron, or selenium can exist in the absence of electrocardiographic, morphometric, or humoral (anemia) indices. Pronounced subcellular alterations are apparent at six weeks post weaning in either dietary copper or iron restricted rats. The onset of selenium deficiency intracellular cardiomyopathy appears to be more imperceptible at six weeks whereas extracellular alterations are more evident.
References


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CHAPTER IV

CARDIOMYOPATHY IN THE PIG FED A COPPER-RESTRICTED, HIGH-ZINC DIET

Abstract

Pigs were made copper-deficient by feeding a copper-restricted, high-zinc diet over an eight week period and compared to copper-adequate, high-zinc diet fed pigs. Cardiac effects were examined in regard to gross morphometry and ultrastructure of the heart, electrocardiography, and lipid profiles. The copper-restricted diet fed pigs exhibited a marked deceleration of growth and lower serum and liver copper concentrations in comparison to the copper-adequate diet fed pigs. The copper-restricted diet fed pigs had a significantly greater heart weight % of body weight and left ventricular free wall width:body weight ratio, both evidence of hypertrophy. Electrocardiography showed a protracted QT interval after 8 weeks of diet consumption in the copper-restricted diet fed pigs. Augmented mitochondrial volume density and mitochondria:myofibril volume density ratio were observed in the copper-restricted diet fed pigs along with excessive lipid and glycogen inclusion. Degradation of Z-
lines, intercalated disc, and sarcomeres were also evident in the copper-restricted diet fed pig micrographs. Some focal regions of disorganization and augmented glycogen deposition were observed in the copper-adequate diet fed pig micrographs, however, they were minor in comparison to the general disorganization and degradation of the copper-restricted diet fed pig micrographs.
Introduction

The development of cardiomyopathy is rapid and pronounced in animals fed a diet restricted in the essential trace element copper (1-5). Copper-deficiency cardiomyopathy (CuD-Cmyo) is manifested by alterations both in the heart and the blood. Alterations are observed electrocardiographically (1-3,6-8) and in ventricular pressure development (1,9,10). The heart tissue itself develops gross hypertrophy as evident by an augmented absolute heart weight (4,7,8,10,11,13) and heart weight:body weight (4,7,8,10,11-13). The cardiomegaly is an expression of alterations at the subcellular level as the enlarged cardiomyocytes exhibit an increased mitochondrial compartment (2,5,7). Increased lipid deposition (2,7,9), decreased copper content (6), excessive perivascular collagen deposition (7,9) and disruption of basal laminae dimension and continuity (7,9) are also characteristic of CuD-Cmyo myocardium. Changes in blood composition include anemia (4,8,10,14,15), hypercholesterolemia (6,11,15,16), hypoceruloplasminemia (10,17,18) and reduced blood plasma copper (18,19).

The above studies compiled to characterize CuD-Cmyo utilized the rat, a species with cardiac anatomical and blood lipid profile dissimilarities to the human. The pig, on the other hand, offers a more conserved cardiovascular model to the human (20,21). Investigation into the effects of dietary copper-restriction upon the pig heart are not as numerous as
the rat, but do suggest many common developed copper-deficient cardiac pathologies to those reported in the rat. Shields et al. (22) observed hypocupremia, hypoferremia and a decreased volume of packed red cells (VPRC) in the blood of copper-deficient pigs who showed an extremely high incidence (96%) of cardiovascular lesions (hypertrophy and aortic lesions) upon death. In a later study, Medeiros et al. (23) observed decreased heart and plasma copper, increased absolute heart weight and heart weight:body weight ratios in copper-deficient pigs. Electron micrographic investigation of left ventricular cardiac myocyte ultrastructure and volume densities revealed abnormal mitochondrial appearance and alterations in myofibrillar and mitochondrial volume densities including an increased mitochondria:myofibril volume density ratio. Other studies of copper restriction (24,25) utilizing the pig also demonstrate similarities in copper metabolism and a conserved CuD-Cmyo between the pig and rat.

Copper bioavailability is effected by many factors including dietary zinc content. An inverse relationship exists between diet zinc composition and intestinal copper absorption and internal copper status (26). Thus a diet restricted of copper and high in zinc should expedite and exacerbate the development of CuD-Cmyo.

The purpose of this study was to further investigate and document the effects of copper-deficiency upon the pig cardiovascular components by examining: 1) the heart gross
morphometry and subcellular ultrastructuring and componentry, and 2) blood lipid profile (serum triacylglycerol, HDL, and cholesterol). The design of the diet was to not only restrict copper but to increase zinc concomitantly to expedite and potentiate the copper restriction.
Materials and Methods

**Animal Diets.** The composition of the diets was formulated to meet or exceed the nutrient requirements of growing pigs (27) (Table 5). Diets were analyzed by flame atomic absorption spectrophotometry for copper and zinc content, as described below, and contained less than 1 mg/kg diet for the copper deficient group (NC (no copper)) and approximately 8 mg/kg diet for the copper adequate group (AC (adequate copper)). The zinc content of the diets was approximately 1000 mg/kg. The diet also contained 120g/kg coconut oil and 10g/kg cholesterol by weight as atherogenic components to the diet.

**Experimental design.** Nursing male Yorkshire pigs (15 days of age and specific pathogen free) were randomly assigned to either a copper-adequate (AC) (n=5) or copper-deficient (NC) (n=5) dietary group so that the mean initial weights were similar. Pigs were individually housed in 24 square foot pens containing stainless steel walls and concrete floors. The pens were maintained between 21-24°C with a 12 hour light/dark cycle. Stainless steel feed dishes and watering pans were utilized. The use of stainless steel was necessary to minimize copper contamination and the influence of other minerals which may be found in other types of housing materials. Deionized-distilled water and diet were provided without restriction and weekly weights were recorded and evaluated for the animals ability to thrive. This protocol was
approved by The Ohio State University Institutional Animal Welfare Committee.

Table 5

_Composition of Copper-Adequate and Copper-Deficient Diets_

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Percentage by weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein(^1)</td>
<td>24.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>29.5</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>22.5</td>
</tr>
<tr>
<td>Coconut Oil</td>
<td>12.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5.0</td>
</tr>
<tr>
<td>AIN-76 vitamin mix(^2)</td>
<td>1.0</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.3</td>
</tr>
<tr>
<td>AIN-76 Trace mineral mix without Cu and Zn(^3)</td>
<td>3.5</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>1.2</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\(^1\)Approximately 80% protein by Kjeldahl analysis
\(^2\)See reference 27 for composition
\(^3\)1g Zn/kg diet and 6 mg Cu/kg (cu-adequate diet only) to be added as carbonate salts. See reference 27 for other mineral ingredients.

At 3, 5 and 8 weeks after the beginning of the study pigs were lightly sedated with ketamine and rhompulin, at species appropriate dosage per body weight, for purposes of restraint and to minimize measurement artifacts. Electrocardiogram traces were obtained as described previously (28) and outlined
below. Blood samples for weeks 3 and 5 were collected in EDTA treated syringes from the jugular vein either two days prior or after ECGs. After eight weeks of consuming the experimental diets and following the last of the above measurements, pigs were fasted overnight, anesthetized (see above), and the thoracic cavity was entered and blood obtained by right ventricular puncture. Pigs were perfused (below) then exsanguinated by severing the jugular vein and hearts and livers were processed as described below.

Hearts were flushed of blood by clamping the severed aortic stump and inserting a syringe through the left ventricular free wall and perfusing under pressure with Ringer’s solution or Delbecco’s phosphate buffered saline supplemented with 14 mM glucose. In vivo whole body fixation followed by perfusion through the hearts with McDowell-Trump’s fixative (2% glutaraldehyde-4% formaldehyde). The hearts were then excised, weighed and bathed with McDowell-Trump’s fixative while a small section of the left ventricular free wall was excised for electron microscopy processing. The hearts were immersed and stored in a container of McDowell-Trump’s fixative. Coronal sections of the hearts were later cut to determine cardiac dimensions.

**Electrocardiograms (ECG).** Lightly sedated pigs were placed in right lateral recumbency. Electrodes forming ECG leads I, II and III were positioned on the limbs and ECG’s were recorded for 1 minute at 25 mm/sec paper speed with 1
cm/mV gain and at 100 mm/sec with 2cm/mV gain. The ECGs were assessed for rate, rhythm, duration (msec) and conventional intervals (PQ, QRS, QT) and amplitudes (mV) of the R wave in lead II. The ventricular axis was determined utilizing all standard leads.

**Heart Dimensions.** Coronal sections were cut and gross morphometric measurements were obtained by calipers utilizing procedures described previously by Jenkins & Medeiros (29). Measurements determined were: width of the left ventricular free wall, right ventricular free wall and the intraventricular septum, apical dimension and left ventricular major length and left ventricular lesser diameter. Ratios calculated were: left ventricular free wall: left ventricular volume and left ventricular free wall: body weight.

**Tissue preparation for transmission electron microscopy analysis.** After perfusion with McDowell-Trump's fixative small samples (<1 mm) of the left ventricular free wall were obtained while the heart was immersed in 2% glutaraldehyde solution. Preparation of the tissue samples for TEM followed the procedure described previously (30) utilizing a series of acetone dehydrations and Spurr resin embedment. Embedment molds were allowed to polymerized in a vacuum oven at 60°C overnight. The resin-tissue blocks were then thick sectioned to ensure appropriate vantage of longitudinal myofibers and then thin sectioned with a Diatome diamond knife (Electron Microscopy Sciences, Fort Washington, PA). Thin sections were
caught on Cu grids and examined by TEM (Philips EM 301B, Eindhoven, The Netherlands), at 60 kV, and the negatives developed and printed to desired magnifications.

**Morphometrical analysis of electron microscopy.** Electron micrograph prints were analyzed as described elsewhere (30). Volume densities (\(\mu\text{m}^3/\mu\text{m}^3\)) of mitochondria (VMITO), myofibrils (VMYO) and "other" intracellular material (VOTH) were determined using print enlargements of 18,800 X that followed a point system previously described by Weibel (31) and Steer (32). "Other" intracellular material is that which is not clearly defined as either mitochondria or myofibril. Volume density mean scores for each diet group were calculated from an average score of three micrographs for each of the three pigs in both diet groups.

**Lipoprotein analysis.** Plasma lipoproteins were separated, purified and components analyzed following a procedure described elsewhere (33). Cholesterol levels were determined by the cholesterol oxidase method (Sigma Chemical Co kit, St. Louis, MO) and triacylglycerol and high density lipoprotein (HDL) cholesterol levels were also determined (Sigma Chemical Co kit).

**Biochemical analysis.** Diet and liver copper and zinc were determined by flame atomic absorption spectrophotometry (AAS) as described previously (30). A certified standard reference material, bovine liver 1577a (National Institute of Standards and Technology, Gaithersburg, MD), was also analyzed by
identical methods and results used to determine if recovery exceeds 95%. Plasma was diluted 1:3 or 1:4 with deionized-distilled water and analyzed for copper directly by AAS.

Hematocrit values were determined in heparinized capillary tubes after centrifugation in a microcapillary centrifuge for 5 min. Hemoglobin was determined by dilution of blood sample in Drabkin’s reagent (Sigma Chemical Inc.) whereby hemoglobin, methemoglobin, and carboxyhemoglobin were converted to cyanmethemoglobin which was measured spectrophotometrically at 540 nm.

Statistical analysis. Data were analyzed using a repeated measures General Linear Model (GLM) ANOVA to determine significant differences between the variation of the means calculated from chronological measures utilizing the SAS software (Statistical Analysis Systems, SAS Institute Cary, NC). Nonparametric ANOVA was also employed to determine significant differences in the variation between medians. Tukey-Kramer Multiple Comparisons Post ANOVA test and Dunn’s Multiple Comparison Post-ANOVA test were then applied if differences in variances were determined to be significant for the parametric and nonparametric ANOVA tests, respectively. Student t-test and the Mann-Whitney test were applied to determine the difference between variables obtained only once, at week 8. \( \alpha \) was set a priori at 0.05.

The text will include results and discussion in regard to parametric tests only. Data in the tables will indicate
significant differences determined by both parametric and nonparametric testing.
Results

All pigs but one completed the 8 week diet treatment period. The one fatality, in the NC group, occurred during week 3. The cause of death was determined to be of a congenital nature upon necropsy. The weekly body weight gain (Figure 5) was lower (p<0.05) in the NC pigs and at the end of eight weeks of diet treatment and the NC body weights were only 56% that of the AC pigs (Table 6). Absolute heart weights were not significantly different between the two groups. However, heart weight as % total body weight were significantly greater for the NC animals (p<0.05), indicative of hypertrophy (Table 6).

Liver and serum Cu analysis demonstrated the effect of the diet treatment as they were significantly lower than the AC group (Table 6, p<0.05). Serum triglycerides did not differ between the two treatments nor did liver Zn after 8 weeks. Serum and liver Cu concentrations of the AC pigs (1.92 and 0.41 µg/g, respectively) were lower than levels previously reported (34).

Hematocrit and hemoglobin were significantly lower in the NC group after 8 weeks of diet treatment (Table 7, p<0.05). Serum cholesterol and HDL levels did not vary between the AC and NC pigs throughout the 8 week investigation (Table 7).

Repeated measurement evaluation of ECG lead II (Table 8 and Figure 6) showed significantly shorter duration of the PQ interval and QRS complex for the NC pigs after 5 weeks of diet
Table 6

<table>
<thead>
<tr>
<th></th>
<th>Copper Adequate (AC) (n=5)</th>
<th>Copper Restricted (NC) (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight</strong></td>
<td>27.4 ± 4.3</td>
<td>15.4 ± 3.2</td>
</tr>
<tr>
<td>(kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Heart Weight</strong></td>
<td>151.0 ± 33.9</td>
<td>131.25 ± 34.4</td>
</tr>
<tr>
<td>(g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Heart weight %</strong></td>
<td>0.54 ± 0.07</td>
<td>0.85 ± 0.1</td>
</tr>
<tr>
<td>of body weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Liver copper</strong></td>
<td>1.92 ± 0.4</td>
<td>0.53 ± 0.25</td>
</tr>
<tr>
<td>(µg/g wet)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Liver zinc</strong></td>
<td>1388 ± 140.4</td>
<td>1235 ± 232.9</td>
</tr>
<tr>
<td>(µg/g wet)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Serum copper</strong></td>
<td>0.41 ± 0.13</td>
<td>0.14 ± 0.04</td>
</tr>
<tr>
<td>(µg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Serum TG</strong></td>
<td>50.1 ± 13.5</td>
<td>62.6 ± 14.4</td>
</tr>
<tr>
<td>(mg/dL)</td>
<td></td>
<td></td>
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</tbody>
</table>

1Mean ± SD.
2Significantly different for both parametric and nonparametric analysis (p<0.05).
3Significantly different for parametric analysis only (p<0.05).
Copper-restricted diet fed pigs had a significantly lower body weight after eight weeks.

Figure 5

Weekly Body Weight Change
Copper Restricted was significantly lower (p<0.05).

Figure 5.
Table 7

Repeated Measurements of Blood Parameters at 3, 5 and 8 Weeks of Diet Treatment

<table>
<thead>
<tr>
<th></th>
<th>Copper Adequate (AC)</th>
<th>Copper Restricted (NC)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Week 3:</strong> (n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>42.0 ± 3.6</td>
<td>41.3 ± 6.6</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.7 ± 1.2</td>
<td>13.1 ± 1.8</td>
</tr>
<tr>
<td>Serum Chol (mg/dL)</td>
<td>168.6 ± 61.5</td>
<td>168.6 ± 22.1</td>
</tr>
<tr>
<td>Serum HDL (mg/dL)</td>
<td>55.6 ± 22.3</td>
<td>51.0 ± 7.8</td>
</tr>
<tr>
<td><strong>Week 5:</strong> (n=5)</td>
<td></td>
<td>(n=4)</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>35.4 ± 1.5</td>
<td>34.1 ± 3.0</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>11.4 ± 0.2</td>
<td>11.3 ± 1.2</td>
</tr>
<tr>
<td>Serum Chol (mg/dL)</td>
<td>144.0 ± 43.9</td>
<td>138.3 ± 28.7</td>
</tr>
<tr>
<td>Serum HDL (mg/dL)</td>
<td>43.4 ± 14.8</td>
<td>47.5 ± 16.8</td>
</tr>
<tr>
<td><strong>Week 8:</strong> (n=5)</td>
<td></td>
<td>(n=4)</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>32.4 ± 3.2</td>
<td>14.8 ± 2.6</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>9.5 ± 1.6</td>
<td>3.6 ± 2.3</td>
</tr>
<tr>
<td>Serum Chol (mg/dL)</td>
<td>122.8 ± 30.9</td>
<td>139.6 ± 50.8</td>
</tr>
<tr>
<td>Serum HDL (mg/dL)</td>
<td>36.2 ± 9.6</td>
<td>39.7 ± 20.6</td>
</tr>
</tbody>
</table>

1 mean ± SD
2 significantly different by both parametric and nonparametric analysis (p<0.05)
treatment ($p<0.05$). Interestingly, these parameters where not altered after eight weeks. However, the QT interval of the NC pigs was significantly greater after 8 weeks ($p<0.05$). There were not significant differences in R wave amplitudes at 3, 5, nor 8 weeks of diet treatment. Subjective analysis of the ECG tracing yielded electrical alternans and an interventricular conduction disturbance in one of the NC animals (NC02) at week 5 and week 8, respectively. There was not a difference in mean ventricular axis.

Excised hearts from both groups presented focal points of discolorization on their mitral valves. Gross morphometrical analysis (Table 9) after eight weeks of diet treatments yielded significantly shorter left ventricular major dimensions and greater left ventricular free wall width:body weight ratios for the NC animals. Thus, evidence of hypertrophy is revealed as significant differences in heart weight as a percent of body weight and left ventricular free wall width:body weight. Coronal sections of hearts are displayed on Plate VII.

Ultrastructural assessment after 8 weeks of diet treatment yielded greater mitochondrial volume densities and mitochondria:myofibril ratios for the NC pigs (Figure 7). Focal mitochondrial enlargement, sarcomere disorganization, Z-line disintegration and disruption of intercalated discs were evident in some of the NC micrographs (Plates VIII-X). Also, the NC micrographs displayed increased cellular inclusions of
Table 8

Repeated Measurements of Electrocardiographic Parameters at 3, 5 and 8 Weeks of Diet Treatment\(^1,2\).

<table>
<thead>
<tr>
<th></th>
<th>Heart Rate (beats/min)</th>
<th>QRS (mS)</th>
<th>PQ (mS)</th>
<th>QT (mS)</th>
<th>R wave (mV)</th>
<th>Axis (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Week 3:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC (n=5)</td>
<td>177.2±20.3</td>
<td>52.6±7.6</td>
<td>87.4±5.1</td>
<td>207.2±16.3</td>
<td>0.53±0.16</td>
<td>80.0±12.0</td>
</tr>
<tr>
<td>NC (n=5)</td>
<td>170.7±16.9</td>
<td>46.8±7.4</td>
<td>91.4±3.9</td>
<td>206.8±17.1</td>
<td>0.55±0.18</td>
<td>80.0±13.4</td>
</tr>
<tr>
<td><strong>Week 5:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC (n=5)</td>
<td>132.2±17.2</td>
<td>60.6±5.4</td>
<td>95.7±7.3</td>
<td>258.6±36.9</td>
<td>0.54±0.16</td>
<td>91.0±11.6</td>
</tr>
<tr>
<td>NC (n=4)</td>
<td>150.3±16.3</td>
<td>50.4±3.1</td>
<td>79.8±10.2</td>
<td>251.3±28.2</td>
<td>0.49±0.20</td>
<td>102.5±12.9</td>
</tr>
<tr>
<td><strong>Week 8:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC (n=5)</td>
<td>108.5±19.1</td>
<td>62.2±7.6</td>
<td>108.2±10.3</td>
<td>263.8±25.8</td>
<td>0.53±0.22</td>
<td>98.0±12.4</td>
</tr>
<tr>
<td>NC (n=4)</td>
<td>119.5±11.9</td>
<td>67.0±11.4</td>
<td>108.0±9.8</td>
<td>332.3±51.1</td>
<td>0.52±0.24</td>
<td>85.0±13.8</td>
</tr>
</tbody>
</table>

\(^1\)mean ± SD
\(^2\)QRS, PQ, QT durations and R wave amplitudes were evaluated from lead II; QRS AXIS was determined from all three Standard Leads.
\(^3\)significantly different (p<0.05)
Figure 6

Pig Electrocardiographs

Pig electrocardiographs at week 3, 5, and 8. Protracted QT interval was measured in the copper-restricted diet (NC) group at 8 weeks. NC02 presented electrical alternans at week 5 and an intraventricular conduction disturbance at week 8.
Week 3

AC01 6-28-93
AC03 6-28-93
AC05 6-28-93
AC07 6-28-93
AC09 6-28-93

Week 5

AC01 7-12-93
AC03 7-14-93
AC05 7-12-93
AC07 7-14-93
AC09 7-14-93

Week 8

AC01 8-2-93
AC03 8-2-93
AC05 8-2-93
AC07 8-4-93
AC09 8-4-93

Figure 6.
Figure 6. (continued)
Table 9

**Gross Morphometrical Assessment of hearts after 8 Weeks of Diet Treatment**¹.

<table>
<thead>
<tr>
<th></th>
<th>Copper Adequate (AC) (n=5)</th>
<th>Copper Restricted (NC) (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVFW (mm)</td>
<td>12.88 ± 2.4</td>
<td>10.62 ± 2.0</td>
</tr>
<tr>
<td>RVFW (mm)</td>
<td>7.03 ± 0.7</td>
<td>6.32 ± 1.6</td>
</tr>
<tr>
<td>AD (mm)</td>
<td>3.76 ± 2.2</td>
<td>5.73 ± 1.6</td>
</tr>
<tr>
<td>IVS (mm)</td>
<td>13.07 ± 1.0</td>
<td>12.19 ± 1.9</td>
</tr>
<tr>
<td>LVLD (mm)</td>
<td>30.74 ± 3.8</td>
<td>28.41 ± 4.5</td>
</tr>
<tr>
<td>LVMD² (mm)</td>
<td>59.99 ± 4.4</td>
<td>50.86 ± 3.1</td>
</tr>
<tr>
<td>LVV (cc)</td>
<td>30.09 ± 9.8</td>
<td>24.19 ± 10.2</td>
</tr>
<tr>
<td>LVFW:LVV</td>
<td>4.92 ± 2.8</td>
<td>5.50 ± 3.4</td>
</tr>
<tr>
<td>LVFW:BW² (x10⁻¹)</td>
<td>4.67 ± 0.38</td>
<td>6.98 ± 1.04</td>
</tr>
</tbody>
</table>

¹mean ± SD.
²significantly different for both parametric and nonparametric analysis (p<0.05).
Plate VII

Hearts: Coronal Sections

Coronal sections of copper-adequate (top row) and copper-restricted (bottom row) diet fed pigs. Copper-restricted diet fed pig hearts had a greater left ventricular major dimension ($p<0.05$).
Figure 7
Subcellular Volume Densities

Pig subcellular volume densities at week 8. The copper-restricted diet fed pigs had a greater mitochondrial volume density and mitochondrial volume:myofibril volume density ratio and a lower myofibril volume density (p<0.05). VMITO = Mitochondria Volume Density; VMYO = Myofibril Volume Density; VOTH = "OTHER" Volume Density.
Figure 7.

VMITO, VMYO, and VMITO:VMYO are significantly different (p<0.05)
Plate VIII
Electron Micrographs: Myocyte

Transmission electron micrograph of copper-adequate (A) and copper-restricted (B) diet fed pigs at 8 weeks. The copper-restricted pigs displayed excessive glycogen deposition (arrow) and myofibril disorganization. Bar = 1 μm.
Plate IX

Electron Micrographs: Volume Densities

Transmission electron micrograph of copper-adequate (A) and copper-restricted (B-F) diet fed pigs at 8 weeks. The copper-restricted pigs displayed excessive glycogen deposition (thin arrow), large lipid droplets, increased myofibril spacing, and disrupted myofibrils. M = mitochondria; my = myofibril; S = sarcolemma; Z = Z-line; T = T-tubule; L = lipid droplet. Bar = 1 μm.
Plate IX (continued)
Plate X

Electron Micrographs: Intercalated Discs

Transmission electron micrograph of copper-adequate (A) and copper-restricted (B) diet fed pigs at 8 weeks. The copper-restricted pigs displayed disruption of intercalated discs in many regions of the micrographs. Arrows indicate desmosomes. M = mitochondria; my = myofibril; T = T-tubule. Bar = 1 μm.
glycogen and lipid droplets. The AC pig micrographs were not without some regions of anomaly. Areas of excessive glycogen deposition were also apparent in their micrographs although certainly not to the extent as displayed in the NC micrographs. Also some focal sarcomere disorganization was observed in the AC micrographs.
Discussion

Much of the experimental investigation regarding dietary copper-restriction and cardiovascular manifestations has utilized the rat as the animal model. In the present study we investigated dietary copper restriction in the pig to further characterize pig CuD-Cmyo, a species with a more conserved anatomy and blood lipoprotein profile to the human (21). Results of this study yields supporting evidence to previous copper-restricted pig studies that dietary copper restriction leads to detrimental alterations in the heart. Thus copper restriction not only affects rats, the animal model of most investigations, but also animals with a more similar heart structure and function to humans.

As noted in stating the results, our values for the copper-adequate diet fed pigs liver and serum Cu (µg/g) were below previously reported levels for pigs. A possible explanation for the lower levels of Cu is the augmented level of zinc in the diet. We provided 1000 mg/kg of zinc, in effort to enhance a copper-restriction effect. Zinc decreases intestinal absorption of Cu by up-regulating metallothionein in enterocytes and other tissue (35). Thus, an inverse relationship exists between dietary zinc content with liver and serum Cu and intestinal Cu transport in rats (26,34,35). The effect of a high zinc diet may also explain some of the subtle abnormalities recognized in the copper-adequate diet fed pigs stated in the preceding and current sections.
Copper deficient animals have shown a reduced ability to thrive (2,6,10,12), anemia (2,6,8,10), increased absolute heart weights (2,10,11,23) and heart weight:body weight ratios (2,8,10,12,18,23), organ and blood hypocuproemia (6,8,10,18,23,36), decreased cupro-enzyme activity (2,12), pronounced cardiomyopathy (2-5), and arterial disease, aneurysms and rupture (37). We presently observed a decelerated body weight gain, anemia, decreased copper concentration of serum and liver, and pronounced alteration at the subcellular level.

Alterations in electrocardiographic abnormalities has been observed in copper-deficient animals. Electrocardiographic changes include increased R-wave amplitude (2,16), protracted QRS complex (38) and QT interval duration (2,8), and electrical slurring of isoelectric baseline line (2). In the present study, we observed a shorter PQ interval and QRS complex duration in the copper-restricted diet fed pigs after 5 weeks and a protracted QT interval at 8 weeks. The shorter PQ interval may be a result of a slightly increased heart rate in the copper-restricted pigs. Heart rate and PQ interval share an inverse relationship within normal ranges (28). A positive chronotropic and dromotropic effect upon the heart may be a compensatory mechanism for the reduction in contractility reported previously in copper deficiency (1,9). However, a strong effector of cardiac chronotropy and dromotropy is the catacholamines, epinephrine.
and norepinephrine, which are synthesized by sympathetic postganglionic fibers and adrenal medullary tissue. Dopamine β hydroxylase, a cupro-enzyme is a key enzyme in synthesis process from dopamine. Seidel et al. (39) previously observed reductions in cardiac norepinephrine and increased dopamine in copper-deficient rats. Another possible mechanism may be enhanced thyroid function (T3) in copper deficiency, although not much literature is available in support. However, Oliver (40) reported that copper may be directly related to thyroid function as copper-restriction seemed to enhance thyroid deficiency.

Another interesting electrocardiographic feature of the copper-deficient animal is a protracted QT interval (1,8,9). Hamlin et al (28) developed a regression equation for predicting QT intervals in miniature pigs, showing that as heart rate increases QT interval shortens within normal physiological ranges. We observed a protracted QT interval at week 8 in spite of a slightly greater heart rate in the copper-restricted diet fed pigs. QT interval denotes complete electrical activity of the ventricles as it originates at the first ECG sign of ventricular depolarization and concludes at the end of ventricular repolarization. QRS complex duration at 8 weeks were not significantly different between the two group, thus a larger portion of the protracted QT interval is accounted for by ventricular repolarization. Length of ventricular repolarization is inversely related to the
activities of ion pumps and the re-establishment of a resting membrane potential and pre-excitatory calcium sequestering across the sarcoplasmic reticulum and plasma membranes.

Another observation reported in copper-deficient rats is hypercholesterolemia (6,11,15,16). We did not observe a significant difference in serum cholesterol or HDL cholesterol at any of point during the study. However, it must be recognized that an elevated zinc content in the diet may have effected the development of differences in serum cholesterol. It is recognized that our levels of serum cholesterol are slightly greater than those reported elsewhere for pig (1.37-2.7 mmol/L) (41). This could be an effect of our diet which included 120g/kg coconut oil and 10g/kg cholesterol.

Cardiomegaly with a concentric pattern of myocardial hypertrophy has been documented as a characteristic of CuD-Cmyo (1). Increased absolute heart weights and heart weight:body weight ratios have strongly been documented (4,7,8,10,13). The absolute heart weight was not different between the two copper-adequate and copper-restricted diet groups nor were ventricular dimensions with the exception of left ventricular major dimension and the left ventricular free wall width:body weight ratio. A greater left ventricular free wall width:body weight ratio in the copper-restricted diet fed pigs concomitant with a greater heart weight % of total body weight is indicative of hypertrophy. The heart weight % of total body weight of the copper-restricted diet fed pigs was
well above a regression curve for heart weight % body weight for pigs reported previously (42).

CuD-Cmyo dramatically effects cardiomyocyte subcellular volume densities, organization and componentry of the perivascular compartment (1-5,7,9,17,23). Dramatic subcellular alterations were evident in the micrographs from pigs fed the copper-restricted diet. Enhanced mitochondrial volume density and mitochondria:myofibril volume density ratios have been reported (1,2,5,7,9,23) and are supported by our findings. Sarcomere disorganization with Z-line disintegration seemed to be a general manifestation along with disruption of intercalated discs. Some sarcomere disorganization was apparent in the copper-adequate diet fed pig micrographs possibly attributable to the effects of high amounts of zinc in the diet reducing copper bioavailability.

In conclusion, the CuD-Cmyo in the pig appears highly conserved to the disease state in the rat, following a similar time course as well. Hypertrophy and electrocardiographic alterations along with pronounced subcellular transformations characterize the copper-deficient pig heart.
References


CHAPTER V

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

The impact of bio-deficiencies of the essential trace elements copper, iron, and selenium upon the heart has received much investigative attention. Experimental inquiry into the developed cardiomyopathy associated with each of these elements has mostly been conducted independently. The objective of the first study was to evaluate the similarities and differences of iron-, selenium-, and copper-deficient rat hearts.

Results demonstrated that 6 weeks of dietary restriction for each of the three elements yields subcellular alterations. The copper- and iron-restricted rat electron micrographs displayed increased mitochondrial compartments and mitochondria:myofibril volume densities. There was also alteration of sarcomere organization and excessive lipid droplet inclusion. The selenium-restricted rat heart electron micrographs appeared to display focal regions of discontinuity of mitochondrial bilayers. All three element-restricted group micrographs exhibited alterations of perivascular basal laminae.
There was some indication in all three trace element restrictions that other signs of deficiency were developing. These signs included a decelerated body weight gain, which was evident in all groups and was significant in the iron-restricted group, and electrocardiographic aberrations whereby a trend towards protraction of the QT interval was evident in all groups although not statistically significant. Thus the results of the first study suggest that subcellular alteration is a very early characteristic of all three trace element-restriction cardiomyopathies and occurs concurrent to the other restriction signs associated with the deficiency disease.

Alterations at the subcellular levels and concomitant changes in ECGs, growth, and heart morphometry have been well documented to date. Based upon the current and previous observations of altered cellular and extracellular aspects, hypertrophy, and cardiac failure associated with these element-restrictions the direction of future research efforts may be best served by focusing upon the molecular events of transcription and translation of cellular and extracellular proteins, along with the metabolism and appropriateness of these proteins during the developing deficiency. What are the signal(s) of hypertrophy? What are secondary effects of these signals? Are they physiologically beneficial or pathologically problematic? Furthermore, as the heart serves all tissue in the body, what are distal effects of the trace element
restriction? Also, are the effects within that tissue due to the element deficiency within the tissue or secondary due to primary effects on supportive tissue?

In regard to iron-deficiency alone, some investigators speculate that the stimulus for hypertrophy in the iron-deficient heart is augmented catecholamine interaction with cardiac adrenergic receptors. As tension is increased in chamber walls norepinephrine is released. Also, if an cardiac \( \beta_1 \) adrenergic receptor antagonist is administered in the development of iron-deficiency the hypertrophy is blocked. The next step would be to see if there are increases in norepinephrine catabolites (3,4-Dihydroxymandelic acid (DOMA), 3',4-Dihydroxyphenylglycol (DOPEG), and 3 Methoxy-4-hydroxy-mandelic acid (VMA)) or increased activity of the involved catabolic enzymes (catechol-O-methyltransferase (COMT) and Monoamine oxidase (MOA)).

In regard to copper-deficiency, preliminary efforts have shown the quantity of norepinephrine to be decreased in copper-deficient hearts. This finding goes without suspicion due to the copper constituency within dopamine \( \beta \) hydroxylase, the enzyme responsible for catalyzing the conversion of dopamine to norepinephrine. Thus the mechanism of copper-deficiency hypertrophy may not be regulated through augmentation of norepinephrine interaction with \( \beta \) adrenergic receptors as proposed for iron-deficiency related cardiac hypertrophy. Therefore the reduced cardiac norepinephrine
concentration in iron-deficient hearts may be attributed to increased release while the reduced norepinephrine content found in copper-deficient hearts is more likely due to decreased synthesis. This may help explain why the pattern of hypertrophy is different despite many similarities in both mineral-deficiencies such as anemia and expansion of the mitochondrial compartment.

Interestingly, even though norepinephrine levels are decreased, most investigators report normal heart rates or even accelerated heart rates in copper-deficiency (normo- and positive chronotropy). However, some investigators report protracted PQ and HV intervals which would suggest a negative dromotropic effect during copper deficiency. Furthermore, results from the first dissertation study suggest that a negative inotropic effect may be characteristic of copper-deficient hearts as suggested by a lower LV dP/dt_{max}. It may be that heart rate is not reduced in copper deficiency due to compensatory negative feedback mechanisms elsewhere in the body responding to either a reduced cardiac output, anemia, or some other factor. A common observation of copper-deficiency is a decelerated growth rate. It still remains unclear as to whether all of the body weight difference can be attributed to a reduction in dietary intake, metabolic activities, or a decrease in efficiency. Thyroid hormone would certainly have its effects upon the heart and metabolism of much of the body’s tissue. Also, growth hormone will take upon a more
metabolic role in mammalian physiology if insulin metabolism is hindered.

Protracted selenium-restriction also is associated with cardiac hypertrophy as evident in Keshan Disease. The primary (or secondary) mechanism for this hypertrophy has not been strongly investigated to date.

At this point the copper-deficient heart has been characterized with consistent investigative efforts, however, the mechanisms for much of the remodeling remains speculative. Future efforts in further characterizing this model should include a more mechanistic design.

The objective of the second study of this dissertation investigated the development and characteristics of the copper-deficient pig. Since much of the controlled cardiac investigation pertaining to copper-deficiency utilizes the rat as the model, a further characterization of the cardiac effects due to copper-deficiency in pigs in a temporal fashion was pursued. The design included not only copper-restriction from the diet (< 1ppm), but also an elevated zinc content as well. Excessive dietary zinc upregulates metallothionein transcription in enterocytes which nonspecifically binds divalent cations present therefore reducing copper absorption from exogenous and endogenous sources.

The results of the study suggest that the developed copper-deficiency cardiomyopathy in pigs is very similar to rats. A decelerated body weight gain was accompanied by
cardiac hypertrophy and electrocardiographic alterations. Furthermore, the changes in copper-deficient pig ECGs (protracted QT interval) has been reported often in investigations of the copper-deficient rat heart.

Since the characteristics of copper-deficiency appear conserved between the species, further investigation into the mechanism of development should be the next step in copper-deficiency exploration. Possible areas of consideration include:

2. Are there compensatory mechanisms for reduced norepinephrine, or perhaps the reduced quantity is still enough for acceptable activity? Like many compounds in the body, basal norepinephrine content of cardiac sympathetic postganglionic fibers could be in excess of physiological need.
3. Are cardiac myocyte and autorhythmic cell β adrenergic receptors upregulated in copper-deficiency for greater efficiency?
4. What is the cause of the protracted QT interval. Are K+ channels and Ca"+/ATPase or Na/K ATPase pump activities (or quantity) reduced in copper-deficiency? If so, is this due to transcription, translation or perhaps an interaction with a disorganized basal laminae?
5. What is the effect of a marginal copper-restriction upon
the heart? Does it effect the heart and other aspects in a similar way to copper-restriction but at a protracted time interval? Furthermore, are the effects reversible with copper-repletion.

The last question addresses the notion that the estimated dietary copper intake for Americans is below recommendations and that our normal copper status may be marginally adequate to meet biological copper demands. Furthermore, it is possible to engage in periods of almost complete copper absence from the diet (weight-loss dieting whereby copper is not a component of low-calorie shakes and foods). Thus, will the effects of dietary copper-deficiency during that period be rapid due to poor basal status, and also, will any alterations be reversible?
APPENDIX A

GLUTATHIONE PEROXIDASE ASSAY
Glutathione Peroxidase (GSH-Px) Assay

Tissue preparation

1. At excision, rinse tissue in ice cold 0.9% saline, blot dry, record weight, store on ice or in refrigerator. Store in freezer if assay is not immediate.

2. Prepare liver homogenates utilizing 0.05 M potassium phosphate buffer, pH 7.0 with 0.24 M sucrose. Use approximately 4 volumes of buffer per 1 volume tissue. (Put demo tissue in graduated cylinder and add buffer till 5 parts are made with 4 parts buffer, 1 part tissue). (can be stored frozen)

3. Mix 0.5 mL homogenate and 1.5 mL GSH-Px buffer in pp-tube and centrifuge for 20 min. at 4°C at 10,000 g (7000 RPM in SM-24 rotor); extract supernatant.

4. Place supernatent in polycarbonyl centrifuge tube (Use matched weight tubes filled with buffer to ± 0.01 g. Centrifuge for 1 hour at 4°C at 105,000 g (38,000 RPM in 40.3 rotor). Extract supernatant (enzyme) and store in refrigerator of freezer prior to analysis.

5. Dilute 0.1 mL of supernatent in GSH-Px buffer immediately prior to analysis, hold on ice. (For liver try 15 X dilution of supernatant; heart or kidney try 4 X dilution; for Se- may not need any dilution)

6. Prepare Mix A: mL of each/X# of assays

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>1 assay</th>
<th>25 assays</th>
<th>50 assays</th>
<th>100 assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Buffer</td>
<td>0.4</td>
<td>10.0</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>2) NADPH</td>
<td>0.1</td>
<td>2.5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>3) GSH</td>
<td>0.1</td>
<td>2.5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>4) GSSGR</td>
<td>0.1</td>
<td>2.5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>5) NaN₃</td>
<td>0.1</td>
<td>2.5</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

| | 0.8 | 20 | 40 | 80 |

7. Preparation of the spectrophotometer (Beckman 68)

a. Plug in to turn on, allow 30 minutes to warm-up.
b. press UV (light source)
c. press program the 6 (step function)
d. edit=0 will appear, enter wavelength 320 (with w.l. key)
e. # cells = 6, blanks equal 0, tabulate: yes, initial time 60 (60"), T time 2 (2 min), 1 cycle, span: 1, 0=cal. results:yes
8. Enzyme assay: Place 0.8 mL of Mix A in 1 mL cuvette; add 10 to 100 μL enzyme (add 0-90 μL buffer to make 100 μL); mix; place cuvette in spectrophotometer for 2 min; add 0.1 mL HP (substrate); mix; (final volume= 1.0 mL); record the change in absorbance from 0-4 min at 1 min intervals.

9. Blank: substitute GSH-Px buffer for enzyme; subtract blank from sample in calculations

Check for linearity of reaction with time and concentration

CALCULATIONS

1 unit of enzyme activity is defined as: 1 μmole of NADPH oxidized/min. Typically expressed as Units/mg protein. The molar extinction coefficient (E) for NADPH is 6,220 L/mole or 6.22 mL/mumole

\[ a = Ebc; \quad c = a/Eb; \quad a = \text{change in ABS/min (corrected for blank(B))}; \quad c = [\text{NADPH}] \text{ oxidized} \]

1) \[ \text{μmole NADPH oxidized/min/mL enzyme solution} = \frac{(\Delta \text{ABS/min} - B) \times \text{dilution of enzyme}}{6.22 \times \text{mL enzyme solution in assay}} \]

2) \[ \text{mg protein/mL} = \frac{\text{ABS Lowry} \times \text{Slope Lowry} \times \text{dilution of enzyme for Lowry}}{\text{mL enzyme used for Lowry} \times 1000} \]

3) \[ \text{μmole NADPH oxidized/min/mg protein} = \frac{[\text{μmole NADPH oxidized/min/mL enzyme solution}]}{[\text{mg protein/mL enzyme solution}]} \]

REAGENTS: (use ddH₂O for all reagents)

1. GSH-Px buffer: Potassium Phosphate Buffer (pH=7.4; 0.05 M) with EDTA (0.005 M):

a) Add 6.86g KH₂PO₄ (MW=136, acid) and 1.68g Na₂EDTA (MW=336.2) to 1 L ddH₂O. (can be stored at 0-5 C for up to 6 weeks)

b) Add 8.70g K₂HPO₄ (MW=174, base) and 1.68g Na₂EDTA to 1 L ddH₂O.
(can be stored 0-5 C for up to 6 weeks)
c) Put 900 mL base in a 2 L beaker with magnetic stir bar; slowly add acid until pH=7.4. (can be stored 0-5 C for up to 6 weeks)

- will use 0.4 mL/assay

d) Put the remaining 100 mL base in a 500 mL beaker, adjust to 7.0 with acid. Add sucrose to yield 0.24 M sucrose, readjust the pH. This will be used above in the homogenate production (can be stored 0-5 C for up to 6 weeks).

2. NaCl (0.9%): add 9 g NaCl to 1 L ddH2O (can be stored 0-5 C for up to 6 weeks)

3. NADPH (2 mM): use 0.1 mL/assay (can be stored frozen)
   - 27 assays: mix 2.75 mL buffer with 5.5 uM (5 mg) NADPH
   - 54 assays: mix 5.5 mL buffer with 11 uM (10 mg) NADPH.

4. Glutathione, GSH (10 mM): use 0.1 mL/assay (prepare fresh)
   - 100 assays: mix 10 mL buffer with 0.0307 g GSH (MW=307.3)

5. Glutathione Reductase, GSSGR (10 U/ml): use 0.1 mL/assay
   - 27 assays: mix 2.65 mL buffer with 0.015 mL GSSGR (2500 U/1.4 mL).

6. Sodium Azide, NaN₃ (10mM): use 0.1 mL/assay
   - 250 assays: mix 25 mL buffer with 0.0163 g NaN₃ (MW=65).

7. Hydrogen Peroxide, HP (2.5 mM); substrate for Se-dependent GSH-Px; use 0.1 mL/assay.
   - 1760 assays: mix 175.5 ml buffer with 0.05 mL HP (30%, 8.8M, MW=34). (this substrate yields high blanks)

General Mechanism for Glutathione Peroxidase
APPENDIX B

SCORING PROTOCOL FOR BASAL LAMINAE INTEGRITY
# Scoring Protocol for Basal Laminae

1. Electron micrographs will be @ ~ 25,000 X  
2. Contrast and resolution shall be optimal  
3. Scoring shall be blind  
4. Micrographs will be scored on a 1-4 scale presented below

<table>
<thead>
<tr>
<th>Score</th>
<th>Description to Receive Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>Basal laminae is intact and presents a uniformed thickness. Sarcolemma is not ballooned outward. No apparent collagen fibrils.</td>
</tr>
<tr>
<td>3.0</td>
<td>Discontinuity of basal laminae in terms of presence and thickness around both myocytes and capillaries.</td>
</tr>
<tr>
<td>2.0</td>
<td>Basal Laminae thickened and disrupted around myocytes and capillaries. Collagen fibrils present and space between myocyte and capillary greater than usual.</td>
</tr>
<tr>
<td>1.0</td>
<td>All features present as in 2.0 score with more pathology and presence of phagocytes.</td>
</tr>
</tbody>
</table>
APPENDIX C

LIVER SUPEROXIDE DISMUTASE ASSAY
Liver SOD Analysis

Preparation of tissue

1. Obtain bucket of ice and label test tubes with sample codes

2. Pipette 5 mL phosphate buffered saline (PBS) into each test tube.

3. Thaw liver samples and obtain a 1 gram sample (accurately record weight). Quickly place tissue in appropriate test tube (in ice) and expeditiously return unused liver to freezer.

4. Homogenize each tissue with the polytron. With clean forceps, remove any caught tissue from the blade region and re-homogenize the tissue (discard if the tissue is connective tissue). Rinse the polytron shaft in between samples with ddH₂O and dry.

5. Do all samples from one treatment together.

6. Place 1 mL of each sample homogenate into a microcentrifuge tube. Add 0.4 mL ethanol-chloroform to each tube and mix by inversion. Using benchtop microfuge, spin samples for 5 minutes.

7. Remove precipitate and discard. Respin supernatant and ethanol-chloroform for another 2-3 minutes. Transfer the supernatant to clean labelled microfuge tubes. Use immediately of store in freezer (for no > 1 month).

Preparation of the spectrophotometer (Beckman 68)

1. Plug in to turn on, allow 30 minutes to warm-up.
   a. press UV (light source)
   b. press program the 6 (step function)
   c. edit=0 will appear, enter wavelength 320 (with w.l. key)
   d. # cells = 6, blanks equal 0, tabulate: yes, initial time 30 (30"), T time 2 (2 min), 1 cycle, span: 1, 0=cal.
      results: yes

Use magnetic stirring rod and stir TAPS buffer for 3-5 minutes to oxygenate. Do not exceed 5 minutes.
Determination of Pyrogallol quantity to add to TAPS

1. Place 1 mL TAPS buffer in each cuvette. Vary the amount of PG added to each cuvette with 1 mL TAPS. Usually PG in quantity range of about 15-40 uL will yield a desired dA (difference in absorbance) of 0.016. Be sure to mixe TAPS and PG well in the cuvette and read immediately.

Example: dA 30 s 0.016

<table>
<thead>
<tr>
<th>Cuvette</th>
<th>PG</th>
<th>dA/30 s</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>0.017</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>0.012</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>0.0133</td>
</tr>
<tr>
<td>4</td>
<td>22</td>
<td>0.0147</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>0.0153</td>
</tr>
<tr>
<td>6</td>
<td>26</td>
<td>0.0171</td>
</tr>
</tbody>
</table>

The correct volume of PG should be between 24 - 26 uL PG, so try:

<table>
<thead>
<tr>
<th>Cuvette</th>
<th>PG</th>
<th>dA, 30 s</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>0.015</td>
</tr>
<tr>
<td>2</td>
<td>24.5</td>
<td>0.0157</td>
</tr>
<tr>
<td>3</td>
<td>24.5</td>
<td>0.0154</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>0.016</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>0.0161</td>
</tr>
<tr>
<td>6</td>
<td>25.5</td>
<td>0.0169</td>
</tr>
</tbody>
</table>

* Therefore the correct volume of PG to add TAPS this day will be 25 uL PG.

Preparation of sample

1. Dilute sample 1:25 with PBS in clean microfuge tube and store unused homogenate to freezer (960 uL PBS, 40 uL sample)

2. Determine sample volume that will reduce dA by 50 % (from 0.016 to 0.008).

3. To each cuvette, add: - 2 mL TAPS
   - determined volume PBS
   - varying amounts of diluted sample (usually 20-35uL AC, 50-99 uL NC)

Calculations

\[
\frac{V_c}{V_s} \times D_s \times 1.4 \frac{W_s + 5}{W_s} = U_{SCD}
\]

\(V_c\) = total volume in the cuvette (TAPS, PG, sample)
\(V_s\) = volume of sample yielding a 50 % reduction
\(D_s\) = dilution factor (25)
\(W_s\) = weight of original liver sample (approx. 1.0 g.)
Reagents

1. TAPS buffer, 200 mL
   2.43 g TAPS
   75 mg Detapac
   pH to 8.2 using 3 N NaOH and most of water
   Bring up to final volume of 200 mL with dd H₂O
   Store at room temperature for no greater than 30 d.

2. 3 N NaOH, 1L
   120 g NaOH pellets
   Bring up to 1 L with dd H₂O

3. PG, 40 mL
   20 mg PG
   40 mL 0.01 N HCl
   Mix well, stored double sealed in dark bottle, no > 3d

4. 0.01 N HCl, 1 L
   86 mL stock HCl
   Bring up to volume with dd H₂O

5. Ethanol-chloroform, 40 mL
   25 mL 100 % ETOH
   15 mL chloroform
   Store in freezer
APPENDIX D

TISSUE PREPARATION FOR AND OPERATION OF THE ELECTRON MICROSCOPE, PROTOCOL SHEET FIXATION & DEHYDRATION, CHECKLIST FOR MATERIAL NECESSARY TO OBTAIN TISSUE, PRE-STUDY INVENTORY SHEET
TISSUE PREPARATION FOR ELECTRON MICROSCOPY

1. Animals are placed in CO₂ chamber until unconscious yet alive. Check for pedal response to pain and pressure stimuli. Turn off gas.

2. Open thoracic cavity with surgical scissors by pulling up on fur layer to prevent trauma to internal organs. - cut opening then \( \approx \) 1 to 1\( \frac{1}{2} \)" right & left lateral - cut craniad and into ribcage (thereby collapsing lungs via pleural space disruption.

3. Obtain Hct via cardiac puncture into right or left ventricle (preferably right to avoid trauma to desired tissue). Use about 5cc syringe and remove needle (avoid lysing cells) before gently transferring to heparinized tube.

4. With heart still intact and functioning, inject 5 cc 1.0 M KCl into right ventricle (relaxing animal in diastole - look to see if extremities spasm and relax).

5. Heart is excised and perfused (aortally) with Delbuccos to remove blood, then transfer to beaker of Delbucco’s and gently squeezed.

6. Gently shaken and patted and transferred to pre-tared beaker of Delbucco’s for weight. **RECORD WEIGHT**

7. Transfer to first fixative, 2.0 % glutaraldehyde in 0.1 M Sorenson’s in 0.1 M sucrose, transfer to petri dish containing fixative and rinse the heart constantly with fixative.

8. Heart is cut transversely and then cut again to recover a 1 mm thick cardiac doughnut. Samples of the left ventricular wall are cut tangentially to the outer wall to obtain fiber in the longitudinal plane. The pericardium is to be unscathed.

9. Transfer about ten < 1mm cubes into fixative, marking time, and seal.

10. begin series of post fixatives, dehydration series and then embedding resin.
Operation of the Electron Microscope 301B

* the EM is always on.
* don't touch C1
* we use 60 kV
* always turn the IN USE light on

A. Load the Grid (putting in specimen)

1. C2 to 26 with fine inner knob turned down, MAG to tap 1, Filament to 0, pull out empty specimen holder;
2. set rod down upright with button side down, open specimen box and gently obtain desired grid with EM forceps;
3. using EM tool open grid holder and place grid in holder shiny side down, tap wire cover gently to ensure complete clamping of grid, place tool back in petri dish and close grid box;
4. slide loading rod into EM column at 1 o'clock position and wait for PVL light to go off; Turn to 12 o'clock position and wait for PVL & PV1 lights to extinguish;
5. Once vacuum light goes off, push ON button for KV (set at 60 kV) and saturate your filament; Turn up C2 slowly to 17 moving close to crossover, turn up your filament knob slowly until you see a reasonable amount of light, using small knob in the center of C2 to get the smallest image and then center with the deflection knobs; increase the filament knob again until image of filament disappears (donut shape), usually (lately) about 8;
6. Finish loading the specimen by turning C2 back to 26, so your specimen is not hit by an intense onslaught of electrons upon entering)
7. Turn loading rod counter-clockwise to 6 o'clock position and allow to move into EM on its own;
8. Once it has stopped, gently slide rod in as far as it will go and push center button (disengages the specimen holder from rod);
9. Pull rod out slightly and release button and then turn clockwise to 12 o'clock position; rod stays here, specimen is loaded;
10. You can now turn up your condenser C2 to about 17 until the image appears
11. Focus individual eyepieces, one at a time closing other eye during, bring up pointer arrow to help focus using the edges, small screen must be down to focus eyepieces
12. Use translation knobs to move specimen about, pick the appropriate tissue area, adjust magnification (final magnification / 2.5) focus with small screen down using coarse focus buttons and fine focus knob.
B. Taking negatives

1. Once area is focused at correct tap magnification, push dial button to EXP to gage exposure meter. You want a reading of about 70-72.
2. Turn off external light sources and panel lights
3. Lift large screen with right side lever and press the now lit EXP light on left side.
4. Wait until EXP illuminates again and bring down your large screen
5. Record the photo number (# on dial after the exposure), tap and negative magnification, specimen number and comments
6. There are 16 exposures per film box.

C. Grid Removal

1. Spread beam (C2 to 26), Mag to tap 1
2. Turn loading rod anti-clockwise to 6 o’clock and allow rod to slide in while depressing the center thumb button to ensure an open clamp
3. When rod has ceased movement, push center button to open clamp, gently push rod in till it stops and release the button (you have now grabbed the specimen holder)
4. After inserting rod, release thumb from center button
5. Pull back, turn clock-wise to 12 o’clock and pull out of scope

* If your high tension (Kv) goes off during changing of grids, remember your filament setting (about 8), turn it down to 0, spread your beam C2 to 26, turn Kv back to ON (may have to push x2 till 60 illuminates again) and bring filament back to 8.

5. Follow Grid Loading 1-4 then 6-7 for loading, you need to repump the vacuum for each change
6. Bring C2 beam back up to about 17 and refocus eyepieces if necessary

D. Finish Session or Film Change

1. Retrieve specimen as described above and place rod with empty grid holder back in column (must be pumped down) return rod first to 1 o’clock (wait for PVL light to go off) and then to 12 o’clock and wait for the PVL & PV1 lights to go off.
2. C2 to 26 (also turn down inner dial of C2), MAG to tap 1, KV pushed off (out), Filament turned anti-clock wise to 0;
3. If finished, log off in book, check for proper recording of negatives shot
E. Changing Film

1. Pull out center knob between drawers (about 2 cm till a click)
2. Turn clockwise till stops and pull out to vent, leave for \( \geq 1.5 \) minutes.
3. Face the vacuum oven and vent it by lifting tap lever, allow dial to return to zero;
4. Remove loaded cassette with open bottom and film, and take empty cassette (smooth bottom) from shelf. Close oven, close lever and hold cover on oven while pumping vacuum pressure to \( \pm 20 \)
5. Right drawer: turn large under-knob so that the flat side faces forward, open spring lever, open drawer and take out empty cassette and replace it with loaded cassette from oven. Close drawer, move lever back into locking position and turn under-knob back towards scope
6. Left drawer: repeat # 5 but replace the loaded cassette (immediately wrapping lengthwise with gumband) with empty smooth bottom cassette
7. Turn center knob clockwise to stop, wait for PVL light to go out (pumping), push center knob in and turn clockwise till the click (180)
8. Wait for pumping lights to go out before filament can be turned on again (if continuing)
9. Reset number counter in right side slide panel drawer

E. To Take Negatives Out

1. Take negatives to dark room, turn on IN USE sign, turn off inside lights and turn on inside developing light. Replace used film in slide cassettes with fresh film (when replacing, set film leaning in film box with notch in top side upper right corner, this places the appropriate face of film upwards). Notched end in holder springs into encasement and lays one on top of another in cassette with spring bottom
2. Put negatives in personal box (cardboard cover so you can reach in) or develop straight away
3. When finished with negatives, put full cassette and film box back into vacuum oven and repump pressure to 30. Place empty cassette (smooth bottom) on shelf.

F. Developing Negatives

1. Open tap and let water bath fill up (upper 4 extention nozzle)
2. Remove lids on fixer and developer
3. Check temperature of developer, read time needed for temperature from chart (temperature of developer/fixer controlled by temperature of water beneath tanks)
4. Temperature should be 69 F for developer (run water if greater)
5. Turn on gaseous N
6. Load negatives into plastic racks, careful not to touch (start at the 2nd place on rack)
7. Lower plastic rack into developer with N switch down, then switch up when it is in (sends N into developer in bursts)
8. Set timer for desired time (from chart), when finished, remove rack and place in water (if 69°F, then 3:45, follow temp/time chart)
9. Set timer for 1.5 minutes then remove rack and place in other tank fixer for 3.0 minutes
10. Place rack in water bath (large sink) for 20 minutes (lights can be switched on. Turn N and tap off.
11. Remove plastic racks from water bath, rinse off with ddH₂O & place in oven, switch on and set timer for 30 minutes
12. Place negatives in small glassine envelopes and label.
TISSUE EMBEDMENT DATA SHEET

Date ........
Subject & vial number
# ___________
# __________
# __________
# __________

Schedule:
Fixative and Rinse Time (min)
gluteraldehyde fixative........... 60-90
(post sacrifice)
Sorensons rinse buffer............ 10
Sorensons rinse buffer............ 10
Sorensons rinse buffer............ 10
(agitating gently q. 3 ‘)
osmium tetroxide fixative......... 60
(aging gently q. 5 ‘)
Sorensons rinse buffer............ 10
Sorensons rinse buffer............ 10
Sorensons rinse buffer............ 10
(aging gently q. 3 ‘)

Dehydration sequence (2 changes each time)
30% acetone. ...................... 10
50% acetone. ...................... 10
70% acetone. ...................... 10
80% acetone. ...................... 10
85% acetone. ...................... 10
95% acetone. ...................... 10
100% acetone...................... 10
(100% acetone is changed 3 times)
(aging gently q. 3 ‘)

Spurr resin infiltration
1:1 spurr in 100% acetone........ 45-60
spurr resin....................... 60
spurr resin....................... 60
spurr resin....................... 60
(aging gently q. 15 ‘)
(can sit overnight)

Embedment and orientation ..........................................
Polymerization, 60 C, overnight or till hard
Checklist for obtaining samples

- 1.0 M KCl (5 cc/rat)
- Delbucco's (D-PBS) (about 300 mL/treatment)
- 2.0 % glutaraldehyde in 0.1 M Sorenson's rinse buffer in 0.1M sucrose (about 100 mL/treatment)
- disposable pipettes
- dissecting tools (surgical scissors, forceps, scalpel)
- 5 cc syringes (4/rat) (1/reagent + 1 for Hct)
- needles (green and pink) (check compatibility with syringes)
- marker
- tape
- petri dishes
- tri-pour beakers
- tin foil (organ save)
- double edged razor blades (3/rat)
- wooden stand with marked glass vials with plastic lids (2/rat)
- 2cc tubes with heparin for blood
- gloves (1-2 pair/animal)
- dd H₂O in rinse bottle
- scale
- cooler with ice for organ and tissue salvage
- wooden applicator sticks
PRE-CHECKLIST OF REAGENTS

__ Spurrs resin
__ Acetone stock
__ OsO4 (refrigerator)
__ Sorenson’s Phosphate buffer solution (A & B)
__ Sucrose
__ Delbucco’s
__ Glutaraldehyde
__ KCl stock

OTHER

__ sharpened scissors
__ wooden sticks
__ gloves
__ syringes and compliment needles
__ heparin and tubes (with caps)
__ supplies for Hct
__ razors and/or sharpened scalpel
__ petri dishes
__ pipettes
__ reserve time under hood
APPENDIX E

DATA SHEETS FOR ELECTROCARDIOGRAPHY, dP/dt AND GROSS MORPHOMETRY MEASUREMENTS
ECG Data Sheet

Animal : LEAD : Date:

Paper speed ___ mm/sec
Heart Rate ___ b.p.m.

Waves & Intervals :

P wave (ms) _______
QRS complex (mS) _______
T wave (mS) _______
PQ interval (mS) _______
QT interval (mS) _______

QRS vector axis & subjective comments:

Amplitudes & Ratios

P wave (mV) _______
Q wave (mV) _______
R wave (mV) _______
S wave (mV) _______
T wave (mV) _______

R:S ratio _______

Animal : LEAD : Date:

Paper speed ___ mm/sec
Heart Rate ___ b.p.m.

Waves & Intervals :

P wave (ms) _______
QRS complex (mS) _______
T wave (mS) _______
PQ interval (mS) _______
QT interval (mS) _______

QRS vector axis & subjective comments:

Amplitudes & Ratios

P wave (mV) _______
Q wave (mV) _______
R wave (mV) _______
S wave (mV) _______
T wave (mV) _______

R:S ratio _______

Animal : LEAD : Date:

Paper speed ___ mm/sec
Heart Rate ___ b.p.m.

Waves & Intervals :

P wave (ms) _______
QRS complex (mS) _______
T wave (mS) _______
PQ interval (mS) _______
QT interval (mS) _______

QRS vector axis & subjective comments:

Amplitudes & Ratios

P wave (mV) _______
Q wave (mV) _______
R wave (mV) _______
S wave (mV) _______
T wave (mV) _______

R:S ratio _______
**dP/dt**

**DATA SHEET**

**DATE:**
Animal group & #: Calibration: \( \text{mm} = \text{mmHg} / \text{s}^{-1} \) Verify:

**Left Ventricle**

\(+dP/dt\) \( \text{mm} \) \( \rightarrow \) \( +dP/dt \) \( \text{mmHg} \) \( \text{s}^{-1} \)
\(-dP/dt\) \( \text{mm} \) \( \rightarrow \) \( -dP/dt \) \( \text{mmHg} \) \( \text{s}^{-1} \)

peak systolic dP \( \text{mm} \) \( \rightarrow \) \( \text{mmHg} \)

**Right Ventricle**

\(+dP/dt\) \( \text{mm} \) \( \rightarrow \) \( +dP/dt \) \( \text{mmHg} \) \( \text{s}^{-1} \)
\(-dP/dt\) \( \text{mm} \) \( \rightarrow \) \( -dP/dt \) \( \text{mmHg} \) \( \text{s}^{-1} \)

peak systolic dP \( \text{mm} \) \( \rightarrow \) \( \text{mmHg} \)

Comments:

---

**Animal group & #:**

**Calibration:** \( \text{mm} = \text{mmHg} / \text{s}^{-1} \) Verify:

**Left Ventricle**

\(+dP/dt\) \( \text{mm} \) \( \rightarrow \) \( +dP/dt \) \( \text{mmHg} \) \( \text{s}^{-1} \)
\(-dP/dt\) \( \text{mm} \) \( \rightarrow \) \( -dP/dt \) \( \text{mmHg} \) \( \text{s}^{-1} \)

peak systolic dP \( \text{mm} \) \( \rightarrow \) \( \text{mmHg} \)

**Right Ventricle**

\(+dP/dt\) \( \text{mm} \) \( \rightarrow \) \( +dP/dt \) \( \text{mmHg} \) \( \text{s}^{-1} \)
\(-dP/dt\) \( \text{mm} \) \( \rightarrow \) \( -dP/dt \) \( \text{mmHg} \) \( \text{s}^{-1} \)

peak systolic dP \( \text{mm} \) \( \rightarrow \) \( \text{mmHg} \)

Comments:

---

**Animal group & #:**

**Calibration:** \( \text{mm} = \text{mmHg} / \text{s}^{-1} \) Verify:

**Left Ventricle**

\(+dP/dt\) \( \text{mm} \) \( \rightarrow \) \( +dP/dt \) \( \text{mmHg} \) \( \text{s}^{-1} \)
\(-dP/dt\) \( \text{mm} \) \( \rightarrow \) \( -dP/dt \) \( \text{mmHg} \) \( \text{s}^{-1} \)

peak systolic dP \( \text{mm} \) \( \rightarrow \) \( \text{mmHg} \)

**Right Ventricle**

\(+dP/dt\) \( \text{mm} \) \( \rightarrow \) \( +dP/dt \) \( \text{mmHg} \) \( \text{s}^{-1} \)
\(-dP/dt\) \( \text{mm} \) \( \rightarrow \) \( -dP/dt \) \( \text{mmHg} \) \( \text{s}^{-1} \)

peak systolic dP \( \text{mm} \) \( \rightarrow \) \( \text{mmHg} \)

Comments:
### Gross Morphometrics Data Sheet

<table>
<thead>
<tr>
<th>Study</th>
<th>Subject:</th>
<th>Date:</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVFW</td>
<td>Body weight(BW):</td>
<td>______</td>
</tr>
<tr>
<td>RVFW</td>
<td>Heart weight(HW):</td>
<td>______</td>
</tr>
<tr>
<td>AD</td>
<td>HW:BW ratio</td>
<td>______</td>
</tr>
<tr>
<td>IVS</td>
<td>Calculated LVV:</td>
<td>______</td>
</tr>
<tr>
<td>LVLD</td>
<td>LVFW:LVV ratio:</td>
<td>______</td>
</tr>
<tr>
<td>LVMD</td>
<td>LVMD:LVLD ratio:</td>
<td>______</td>
</tr>
</tbody>
</table>

Subjective appearance of the heart in regard to chambers, papillary muscle, chordae tendenae, lesions, valves, folding of the wall, etc:
APPENDIX F

ABSTRACT OF STUDY PRIOR TO DISSERTATION
Male weaning Long-Evans rats were fed diets that were either adequate in copper and selenium (Cu+/Se+) or restricted in either Cu (Cu-) or Se (Se-) for eight weeks. At week 8 electrocardiograms (ECG) and dP/dts were obtained from all but two rats from each group where heart tissue was utilized for electron microscopy.

After ECG recording, the hearts, liver and blood were removed for gross morphometrical measurements, SOD and glutathione peroxidase activity and hematocrit/hemoglobin levels, respectively. Cu+/Se+ rats had a greater body weight than the Cu- and Se- groups. Cu- rats were anemic, exhibited a greater heart:body weight and developed concentric hypertrophy characterized by greater thickness of left and right ventricular free walls, and interventricular septum. ECG recordings from leads aVF and V3 revealed a greater R wave amplitude, protracted QRS segment and QT duration in the Cu- group. Se- rats recorded a greater left ventricular +dP/dt max than both the Cu+/Se+ and Cu- groups.

Myofibril volume densities were decreased in both Cu- and Se- rats in comparison to the Cu+/Se+ rats while Cu- rats showed a greater mitochondria:myofibril ratio than the Cu+/Se+ rats. Evidence of left ventricular free wall myofibrillar disarray was also apparent in both the Cu- and Se- groups. The Se- myofibrils appeared swelled with a greater spacing between organelles and contractile elements indicative of a edema. Also evidence of mitochondrial fragmentation was apparent.

The results suggest that similarities exist in the cardiac remodeling processes associated with copper and selenium restrictions as evident by subcellular alterations.
APPENDIX G

DATA FROM PIG ECHOCARDIOGRAPHY PILOT
### Pig Echocardiography Measurements

<table>
<thead>
<tr>
<th></th>
<th>Week 3 (n=5)</th>
<th></th>
<th>Week 5 (n=4)</th>
<th></th>
<th>Week 8 (n=4)</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>AC</td>
<td>NC</td>
<td>AC</td>
<td>NC</td>
<td>AC</td>
<td>NC</td>
</tr>
<tr>
<td>IVSd (cm)</td>
<td>0.53 ± 0.06</td>
<td>0.54 ± 0.1</td>
<td>0.67 ± 0.09</td>
<td>0.62 ± 0.06</td>
<td>0.78 ± 0.21</td>
<td>0.78 ± 0.27</td>
</tr>
<tr>
<td>LVIDd (cm)</td>
<td>2.93 ± 0.4</td>
<td>2.22 ± 0.5</td>
<td>3.18 ± 0.4</td>
<td>3.27 ± 0.32</td>
<td>3.59 ± 0.98</td>
<td>4.25 ± 0.68</td>
</tr>
<tr>
<td>LVPWd (cm)</td>
<td>0.42 ± 0.05</td>
<td>0.54 ± 0.15</td>
<td>0.62 ± 0.22</td>
<td>0.54 ± 0.12</td>
<td>0.65 ± 0.09</td>
<td>0.75 ± 0.27</td>
</tr>
<tr>
<td>IVSS (cm)</td>
<td>0.70 ± 0.09</td>
<td>0.84 ± 0.20</td>
<td>0.84 ± 0.29</td>
<td>0.89 ± 0.19</td>
<td>1.10 ± 0.29</td>
<td>1.02 ± 0.18</td>
</tr>
<tr>
<td>LVIDs (cm)</td>
<td>1.99 ± 0.43</td>
<td>1.55 ± 0.53</td>
<td>2.18 ± 0.25</td>
<td>2.22 ± 0.27</td>
<td>2.39 ± 0.85</td>
<td>2.78 ± 0.81</td>
</tr>
<tr>
<td>LVPWs (cm)</td>
<td>0.80 ± 0.02</td>
<td>0.79 ± 0.19</td>
<td>1.02 ± 0.12</td>
<td>1.00 ± 0.19</td>
<td>1.19 ± 0.26</td>
<td>1.18 ± 0.26</td>
</tr>
<tr>
<td>LAd (cm)</td>
<td>2.01 ± 0.19</td>
<td>1.80 ± 0.2</td>
<td>2.45 ± 0.03</td>
<td>2.44 ± 0.32</td>
<td>2.69 ± 0.16</td>
<td>2.71 ± 0.25</td>
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<tr>
<td>FS (%)</td>
<td>32.4 ± 8.0</td>
<td>31.9 ± 13.0</td>
<td>31.1 ± 5.7</td>
<td>32.4 ± 4.9</td>
<td>34.1 ± 10.1</td>
<td>35.5 ± 10.6</td>
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<tr>
<td>EPSs (cm)</td>
<td>0.42 ± 0.04²</td>
<td>0.42 ± 0.08</td>
<td>33.5 ± 0.10²</td>
<td>54.2 ± 0.28³</td>
<td>0.42 ± 0.04²</td>
<td>0.42 ± 0.08</td>
</tr>
</tbody>
</table>

*¹ means ± SD
² n=4
³ n=2
List of References


