EFFECT OF IRON OVERLOAD ON CENTRAL NERVOUS SYSTEM
DEMYELINATION IN TRANSGENIC MICE (B6.CG-TG(THY1-YFP)2JRS/J

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ABSTRACT

THESIS: Effect of Iron Overload on Central Nervous System Demyelination in Transgenic Mice (B6.Cg-Tg(Thy1-YFPH)2Jrs/J).

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A number of neurodegenerative diseases like Multiple Sclerosis, Parkinson’s and Alzheimer’s have been linked with iron accumulation in the brain. Iron plays an important role in neural metabolism. However, mechanisms of neural degeneration in iron overload are complex and not clearly understood. We proposed that iron overload may lead to demyelination in B6.Cg-Tg (Thy1-YFPH) 2Jrs/J mice. These mice express spectral variants of GFP (yellow-YFP) at high levels in motor and sensory neurons. Serum iron levels were significantly higher in experimental versus control animals. Brain and spinal cords were harvested and fixed after 4 weeks of iron dextran injections. Tissue slices were stained with Prussian blue, H&E and fluromyelin for light and confocal microscopy. Immunological profile by Flow Cytometric analysis revealed significantly high numbers of CD3+T cells with no differences in CD4:CD8 ratio. This study indicates that iron overload caused a significant inflammation without demyelination in the CNS.
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CHAPTER 1
INTRODUCTION

An essential mineral in our body, iron plays an important role in carrying oxygen via hemoglobin to all parts of the body. It is a critical element in enzymatic reactions and metabolic pathways and participates in many cellular functions (Bersamin 2004). In addition, during fetal development, iron is one of the most important supplements which pregnant women should have, because adequate amounts of iron during the first few weeks of pregnancy help prevent neural tube defects (NTDs) in the baby. Thus, any abnormal iron levels will have a negative impact on many cellular functions in the body.

From the amount of 3-5 g of iron present in the adult human body, about 80% is involved in life supporting biochemical processes such as erythropoiesis, O2 transport and as an element of enzyme structure. The remaining amount is stored in such protein complexes as ferritin (Szatko et al. 1998). Transferrin is a glycoprotein that is responsible for iron homeostasis in vivo; it maintains the iron pool in the systemic circulation by binding with iron tightly but reversibly (Vikram and Zamboni 2009).
1.1. Food Sources Of Iron

Iron from the diet exists in two different forms, a heme iron and a non-heme iron. The heme form only exists in animal tissues, but the non-heme is found in plant foods. Also, non-heme iron is less easily absorbed by the body than is heme iron (Johnston et al. 2007).

The total amount of iron in the body is controlled. The body loses 1 mg of iron daily from sweat and cells that are shed from the skin and the inner lining of the intestines. In normal adults, only 1 mg of iron is absorbed by the intestines daily from food to replace the lost iron. Because of that, there is no excess accumulation of iron in the body. When iron losses are greater, more iron is absorbed from food (Lee 2007).

Iron is absorbed from food into enterocytes lining the duodenum via the divalent metal transporter (DMT1) (Kotze et al. 2009). Iron must therefore be in the divalent form for uptake. This may be achieved by reduction of Fe$^{3+}$ to Fe$^{2+}$ by vitamin C, or by duodenal cytochrome-b, which is also located on the cell membranes of enterocytes (Kemna et al. 2008),(Figure 1).

Serum iron levels can be measured by the concentration of ferritin when iron deficiency is suspected. However, in iron overload, ferritin should not be the only test used because it increases during infection and inflammation (Knovich et al. 2009). Total iron binding capacity (TIBC) is another test which is required with serum iron level when iron abnormality is suspected. The iron concentration divided by TIBC gives the transferrin (Tf) saturation, which is a more useful indicator of iron status than iron quantity or TIBC alone. In healthy people, 20% - 50% available sites in Tf are used to transport iron. In an iron deficiency, iron is low, but TIBC is increased, and Tf saturation
becomes very low. In an iron overload conditions, such as hemochromatosis, iron may be high and TIBC may be low or normal, causing the Tf saturation to increase (Kotze et al. 2009).

1.2. Iron Overload

Iron overload is characterized by increased iron concentration in the body. Excess of iron in vital organs increases the risk for liver disease (cirrhosis, cancer), heart attack or heart failure, diabetes mellitus, bone diseases, metabolic syndrome. Disruption of iron mechanism control leads to accelerated development of some neurodegenerative diseases such as Multiple sclerosis, Alzheimer’s, and Parkinson’s. Iron overload can be inherited genetically or acquired by receiving numerous blood transfusions such as patients with anemia or thalassemia. In addition, getting iron injections, or consuming high levels of supplemental iron can cause iron overload condition. The most common genetic disorder that results in iron overload is hereditary hemochromatosis (Iron Disorders Institute 2009).

Primary hereditary iron overload conditions such as hemochromatosis and secondary iron overload condition are now being increasingly recognized as worldwide epidemics (Oudit et al. 2004). Primary iron overloads being the least common as compared to majority of the cases due to genetic disorders. Hemochromatosis is associated with progressive iron deposition in different organs, causing failure of their functions. Including heart and endocrine organs, iron overload cardiomyopathies are characterized by marked diastolic dysfunction and increased propensity to develop arrhythmias (Buja and Roberts 1971).
The dominant feature of Hereditary Iron Overload (HIO) is the over absorption of iron which is deposited in tissues and organs causing injury and toxicity that leads to heart disease, diabetes, arthritis, cancer, cirrhosis, impotence and sterility (Kotze et al. 2009).

One of the biggest disadvantages of HIO is that it is hard to diagnose. However, it can be suspected in young people with unexplained mild changes in liver function, abnormal fatigue, right hypochondrial pain, arthritis, diabetes, impotence (particularly in young adults), and unexplained cardiac complaints, particularly if more than one of these symptoms is present (Kotze et al. 2009). According to WHO records, about 2 million people suffer from serious illnesses connected with iron deficiency. However, the effects of iron over supplementation are very seldom noted and are treated by scientists as a case study rather than a disease which should be taught in pre/post diploma courses (Szatko et al. 1998).

1.3. Purpose

To Study The Effects of Iron overload on induction of CNS Demyelination in Transgenic Mice (B6.Cg-Tg(Thy1-YFP)2Jrs/J).

1.4. Hypothesis

We therefore, hypothesized: That Iron Overload will Cause CNS Demyelination in this GFP-Variant of Transgenic Mice (B6.Cg-Tg(Thy1-YFP)2Jrs/J).
CHAPTER 2
REVIEW OF LITERATURE:

2.1. Oligodendrocyte and Myelination:

Glial cells are the supporting cells in the central nervous system and there are many types of these cells and oligodendrocytes are one of them. The scientist Rio Hortega was the first one to introduce this term when he was describing cells that had few processes. The main functions of these cells are to form the myelin sheath. The maturation of myelin sheath starts in the fourteenth week of fetal development. They are small in size and have a high density of cytoplasm and a nucleus. Also, they have a large number of microtubules (25 nm) in their processes that may be involved in their stability (Lunn et al. 1997). Oligodendrocytes are classified based on their size, morphology, and the thickness of the myelin sheath they form (Butt et al. 1995). From small cells supporting the short, thin myelin sheaths of 15–30μ diameter axons (type I), through intermediate types (II and III), to the largest cells forming the long, thick myelin sheaths of one 1–3 large diameter axons (Baumann and Pham-Dinh 2001). The myelin sheath covers most of the axons and it is essential for proper conduction of nerve signals between nerves. It is composed of high content of lipids and low content of water to facilitate electrical insulation of axons (Baumann and Pham-Dinh 2001). The process of
myelination is poorly understood. Researchers have summarized this process into three steps:

(1) The migration of oligodendrocytes to axons that are to be myelinated, and the fact that axons and not dendrites are recognized.

(2) The adhesion of the oligodendrocyte process to the axon.

(3) The spiraling of the process around the axon, with a predetermined number of myelin sheaths and the recognition of the space not to be myelinated, i.e., the nodes of Ranvier (Baumann and Pham-Dinh 2001).

The myelination differs from one species to another. For example, in mice it starts at birth in the spinal cord. In brain, myelination is achieved in almost all regions around 45–60 days postnatally. In humans, the peak of myelin formation occurs during the first year postnatally, although it starts during the second half of fetal life in the spinal cord. It can continue until 20 years of age in some cortical fibers, especially associative areas (Bartzokis et al. 2007).

2.2. Iron and neurodegenerative diseases:

Iron is important for CNS physiology as it acts as a cofactor for enzyme cascade of neural metabolism and ATP production (Vikram and Zamboni 2009). Neurons are highly polarized, while proteins of neuronal homeostasis and CNS physiology are synthesized in cell bodies and then transported to functional sites at synapses. In cooperation with iron regulatory proteins, ferritin and ferroportin, transferrin maintains the iron requirement under control across neuron transport in the CNS by regulating iron absorption, transport, storage, and utilization by brain cells (Yang et al. 1984; Rouault
2001; Chua et al. 2007). Therefore, iron is required to maintain the normal cellular functions of neurons and to maintain homeostasis.

Iron has been a target for many studies because of its involvement in many neurodegenerative diseases like multiple sclerosis (MS) which is characterized by demyelination of neurons in the central nervous system (CNS). Another reason why iron is a target for many studies is that histological studies of brain tissues of MS patients show that the damaged area of the brain contained abnormally high levels of iron. Based on a published paper by Elseweidy, 2009, the authors fed rats biscuits enriched with ferrous sulphate to have iron overload condition for 10 weeks. They found out that the levels of serotonin had decreased as well as the levels of dopamine. The histological studies of brain slides showed severe meningeal hemorrhage, congestion, and edema which indicate degeneration of parts of the brain (Elseweidy and Abd El-Baky 2008). These findings were attributed by the authors to the Reactive Oxygen Species (ROS). ROS is defined as a variety of molecules and free radicals, which are chemical species with one unpaired electron, derived from molecular oxygen (Turrens 2003).

Free radicals are considered ROS and are dangerous because they destroy and injure the cells. The relationship between iron and ROS is identified by many hypotheses: One of the hypotheses by Sklodowska, 1979, proposed that iron can act as a catalyst of reactions producing excessive amounts of free oxygen and non-oxygen radicals (Sklodowskam 1979).

It is known that among oxygen radicals the most important are the superoxygen radical (O2•) and the hydroxyl radical (OH•) and the presence of those radicals with free
iron leads to increased formation of ROS which leads to destruction of the cells. (Figure 3)

Another hypothesis of the relationship between iron and ROS is by Gordeuk, 1987, who proposed that another pathologic mechanism associated with ionized iron overload is the damage of nucleonic acid structures. Iron overload is assumed to be in a sense responsible for neoplasia, as are some other metals (e.g. copper). The uncontrolled development of neoplastic tissue can be initiated by the free radicals excess acting mutagenically on nucleonic acids (Gordeuk 1987).

Therefore, high levels of serum iron leads to iron deposition in tissues which subsequently induces the formation of ROS which has a destructive impact on the cells. Plus, the excess amounts of iron are deposited in the tissues and cause inflammation and degeneration of cells as seen in Elseweidy’s work.

Many scholars have studied the distribution of iron in the Central Nervous System (CNS) using histological and immunological techniques. The majority of these studies confirmed that oligodendrocytes and myelin are enriched with iron (Levine and Macklin 1990; Levine 1991). The explanation of the high iron concentration in the oligodendrocytes is still unclear. It is suggested that the high concentration is associated with the huge demand of iron in myelin synthesis and production (Levine and Macklin 1990; Connor 1995). Another finding by Beard, 2003, supported this theory. He found that iron deficiency during early postnatal life causes reduction in myelination. We have to differentiate between the iron overload which is due to iron absorption disturbance and
the normal iron levels found in the oligodendrocytes which are required for myelination (Beard et al. 2003).

Many neurodegenerative diseases have been linked to iron accumulation in the CNS. Parkinson disease patients have shown iron deposits in their brain (Kaur and Andersen 2004). In Multiple Sclerosis (MS) patients, an initial report by Craelius et al., 1982, revealed iron deposits in 5 out 5 MS patients. In addition, to the iron deposits punctuated iron deposits were observed in some neurons similar to those seen in Alzheimer’s patients (Levine 1991). These deposits within neurons likely represent cells undergoing degeneration since neural loss is also a pathological feature of MS. Since iron is required for myelination and oligodendrocyte development, any low level of iron should cause reduced myelination which is confirmed by stained iron enriched oligodendrocytes in the white matter of normal and diseased brains (Todrich et al. 2009). However, iron plays a significant role in neurodegenerative diseases; so far it is not clear how iron accumulates in the brain. One of the recent reports by Jeong and coworkers (2009) who believes that iron accumulation is a multistep process which includes dysregulation of the proteins involved in iron influx and sensing of intracellular iron. Iron accumulates in ventral motor neurons secondary to blockage of anterograde axonal transport and increased mitochondrial iron load (Jeong et al. 2009).

Analysis of iron and iron-related proteins in Cerebrospinal fluid (CSF), blood, and MS brain sections, supported the idea that disruption in iron homeostasis in MS leads to development of the disease. However, it is poorly understood if iron accumulation is attributed in MS pathology or merely reflects an epiphenomenon (Khalil et al. 2011).
2.3. Iron deposition and Multiple Sclerosis (MS):

MS is an autoimmune disease that affects the central nervous system and it is characterized by demyelination in the CNS. The multistep mechanism of the disease involves inflammation, demyelination, and neurodegeneration (Compston 2006). As in many neurodegenerative diseases, the histological slides of MS patients brain and spinal cord show high levels of oxidative minerals especially iron (Sayre et al. 2005). In addition to brain and spinal cord, the walls of veins were affected with iron; and histological studies show the peculiar disposition of the iron stores in MS constantly encircling the venous wall (Adams et al. 1989).

The demyelination of CNS is caused by an autoimmune attack on the myelin sheath by macrophages and T cells. It is not clear how this happens, but many mechanisms have been hypothesized, such as genetic predisposition, environmental factors and infections. MS symptoms vary from fatigue, loss of mobility, bladder, and bowel movement dysfunction. Pathologically, the blood brain barrier becomes damaged; T cells and macrophages enter the CNS and engulf oligodendrocytes. Also, the myelin sheath is destroyed and the astrocytes and microglia undergo gliosis while the axons become transected. Data from several pharmacological and biochemical studies have suggested that iron catalyzes the formation of free radicals, which leads to oxidative stress (Levine and Chakrabarty 2004).

One of the ways to study MS is to study the animal model of the disease, which is Experimental Autoimmune Encephalomyelitis (EAE). It is an inflammatory disease of CNS in rats and mice, which is characterized by demyelination of the CNS and paralysis.
A new study by Grant et al, 2003, shows that iron-deficient mice fail to develop autoimmune encephalomyelitis. This data supports the idea that iron has a direct effect on induction of MS in humans. In the same study, the iron overloaded mice developed EAE (Grant et al. 2003).

A new discovery by Dr. Paolo Zamboni has changed the thinking of the scholarly community toward MS. Many scientists had thought of MS as a white matter disease, but Dr. Zamboni found that MS is a blockage of the pathways. So removing the excess iron from the brain and clearing a couple major veins to reopen the blood flow, the root cause of the disease can be eliminated (Zamboni et al. 2009).

This claim was spectacularly supported by the case of an MS patient who underwent MRI. The study showed that the patient had a blockage in the jugular vein. So, this finding was named in the recent description as Chronic Cerebrospinal Venous Insufficiency (CCSVI) (Zamboni et al. 2009).

In a recent study Vikram and Zamboni focused on iron overload and oxidative stress as the main causes that leads to immune response that cause MS (Vikram and Zamboni 2009). From his previous work, Dr. Zamboni thought that there is a link between the inflammatory process activated in the course of chronic venous disorders (CVDs) (Zamboni 2006; Zamboni et al. 2008), and the venous disorders which have been studied in MS (Minagar et al. 2006; Frohman et al. 2006). It is known that a steady laminar shear stress promotes a release of factors from endothelial cells. Also, these factors inhibit coagulation and migration of leukocytes, while simultaneously promoting normal function in endothelial cells whereas a disturbance or, especially, a reversal of
flow direction, one or both factors may promote an inflammatory reaction, and particularly the expression of surface adhesion molecules (Sorescu et al. 2004; Bergan et al. 2006).

If this principle is applied to Dr. Zamboni’s theory, then the gradual slow flow cerebral venous system can be considered a proinflammatory stimulus, potentially contributing to MS multifactorial etiopathogenesis (Zamboni et al. 2007). Also, the expression of surface adhesion molecules on the blood brain barrier enables auto activated T cells and macrophages to cross the barrier. Those cells are responsible for the adhesion, migration, and inflammation which are believed to be the cause for MS (Geppert and Losy, 1999; Frohman et al. 2006; Minagar et al. 2006). Additional studies supporting this idea of stress increases the expression of molecules were done by Sipe et al., 2002, as well as Ke and Qian, 2003, who found that transferrin receptors on the brain capillary endothelial cells are plentiful in brain and spinal cord slides of MS patients (Sipe et al. 2002; Ke and Qian 2003).

2.4. Iron overloads in Alzheimer’s:

Alzheimer’s disease (AD) is believed to be caused by a combination of hereditary, environmental and lifestyle factors. In AD, metabolic imbalance and the resulting oxidative stress are believed to play a major role in disease pathogenesis (Sayre et al. 2005). The most readily distinguishable characteristic of AD is the presence of extra cellular β-amyloid plaques known as senile plaques, and intraneuronal neurofibrillary
tangles (NFT) as well as neurophil threads and selective neuronal loss (Rolston et al. 2008). There are metal binding sites on the proteins that are accumulated in the brain such as Tau protein. Iron, copper, and other forms of metals bind to the proteins (Madesen and Gitlin 2007). When iron is bound to these sites, it generates \( \text{H}_2\text{O}_2 \) by the Fenton reaction and induces amyloid-\( \beta \) aggregation (Castellani et al. 2007). The extra iron which causes overload could come from excessive heme turnover which catalyzes conversion of heme to iron and biliverdin and then to bilirubin which was found to be increased in AD, suggesting heme turnover as a possible source of increased redox active iron (Smith et al. 1994). As in other neurodegenerative diseases, iron accumulation and increased oxidative stress are seen in AD patients. This supports the idea that iron plays a major role in development of this disease.

Our study focuses on the concept of iron overload and if this overload is associated with causing demyelination. If it did cause demyelination, then the animals should show signs and symptoms that mimic the demyelination in EAE an animal model of MS in humans.
CHAPTER 3
MATERIALS AND METHODS

3.1. Animals

The transgenic twelve-week old male mice were purchased from Jackson laboratory (B6.Cg Tg (Thy1-YFPH) 2Jrs/J. These mice express spectral variants of GFP (yellow-YF) at high levels in motor and sensory neurons, as well as subsets of the central nervous system. Additionally, axons are brightly fluorescent all the way to the terminals of the mice. These mice were given three days as an acclimation period to avoid travel stress which might affect the study. Mice were housed at the mice facility in the biology department where food and water were provided by the animal caretaker. Mice were divided into an experimental group and control group (n=7 in each group).

A total of 14 mice divided into an experimental and control groups of 7 mice each were used in this study. All animals were kept in identical environmental conditions. They were fed immediately upon arrival and throughout the study period a standard mice chow (pellets) and water. The study was conducted in just 30 days. All protocols were approved by the Animal Care Committee of Ball State University.
3.2. Iron Dextran Injections for Iron Overload

The experimental group was injected Intraperitoneal (ip) with 100 µl of iron dextran (10 mg iron) per injection (Sigma, St Louis, MO, USA) in PBS (phosphate buffered saline pH= 7.4 ) five days for four weeks. The regime of injection was adapted based on the iron overload studies (Reardon and Allen 1994). Mice were observed for clinical signs of paralysis or unusual movements and scored on a scale of 0-5 as done for EAE mice. After four weeks, mice were euthanized by overdose of Isoflurane (IsoVet). This method is acceptable by the American Veterinarian Medical Association (VAMA) if it is used in small animals weighing less than 7 kg as mentioned in the VAMA guidelines for euthanasia published in June 2007. Mice were placed in a large jar to allow sufficient O2 in the chamber to prevent hypoxia, and cotton gauze soaked with small amount of Isoflurane in the jar. When the mice were knocked out, blood was collected by the cardiac puncture technique where mice were held by the scruff of skin above the shoulders and a 25 gauge needle was inserted directly toward the heart and when the blood started to overflow, the needle was pulled back gently. Chest cavity was opened, and transcardial perfusion was performed through a small incision in the right atrium and inserting a ventricular catheter. First perfusion was with PBS to wash out the blood, followed by 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA. USA). Finally, the brain and spinal cord were carefully dissected followed by overnight fixation in paraformaldehyde. Tissues were washed with PBS and stored in PBS until the sectioning for light and confocal microscopy was performed.
3.3. Measurement of Serum Iron Content

Blood for serum iron analysis was drawn prior to the transcardial perfusion of the heart. Blood was collected in small vials, allowed to clot by standing for 30 minutes, centrifuged for 15 minutes at 1000 rpm to separate the serum. Serum was carefully aspirated using a pipette in storage vials and frozen for later analysis. Serum iron levels were measured using a colorimetric method (Spectrophotometry). This was done to verify that the mice injected with iron dextran had a significantly high level of iron load as compared to sham injected controls.

3.4. Cryosections

Brain and spinal cord tissues were prepared for cryosections by putting them in a cryo mold then filling the mold with OCT (Optimal Cutting Temperature) which is a water-soluble embedding medium for frozen sections. Molds were placed on dry ice to freeze followed by immersing the tissues in liquid nitrogen for extra freezing. Frozen tissue blocks were attached to the cryostat chuck and 15 µm sections were sliced and mounted on the glass slides. Slides were kept at room temperature for 1 hour to allow sufficient time for the sections to adhere firmly to the slides.

3.5. Fluromyelin Fluorescent Myelin Stain

Floromyelin stain was purchased from Invitrogen (Carlsbad, CA, USA). The stain was prepared by diluting the stock solution 300-fold into PBS. Then, sections were
flooded with staining solution for 20 minutes at room temperature. After that, sections were washed 3 times for 10 minutes in PBS, and finally they were adhered on gelatin coated super frost slides (Fisher Scientific), mounted with fluorescent medium with DAPI (4',6-diamidino-2-phenylindole, a nucleus staining dye) and covered with a glass slip.

3.6. Hematoxylin & Eosin (H&E) Staining

H&E staining was performed according to conventional protocols. The sections were dipped in 100%, 90%, 70% and 50% ethanol each for a period of 10 minutes. After washing thoroughly in distilled water, the brain sections were counterstained with hematoxylin for 10 min and eosin for 30 s. Finally, the slides were dried, mounted with xylene-based-medium on regular glass slides (Fisher Scientific, USA).

3.7. Prussian blue Staining

The Prussian blue staining of tissue sections was done according to conventional protocol used for iron localization. The tissue sections were hydrated by PBS for 20 minutes then immersed in equal parts of hydrochloric acid and potassium ferrocyanide which was prepared freshly before use. After 20 minutes, slides were washed with 3 changes of distilled water followed by counter stain with nuclear fast red for 5 minutes. Slides were then rinsed twice with distilled water, dehydrated by 95% and 2 changes of 100% alcohol. Finally, slides were immersed in xylene twice for 3 minutes each. Slides were then mounted with xylene based medium.
3.8. Confocal Imaging

The fluromyelin stained tissue sections from spinal cords were examined under Zeiss LSM 510 UV META Laser Scanning Confocal Microscope equipped with 40X 1.2 NA and Plane-Apochromat 63X/1.4 DIC. This microscope is housed in the imaging facility located in the Cooper Science Building of Ball State.

3.9. Light Microscopy

The slides that were prepared by H&E and Prussian Blue staining were examined and studied under the light microscope. The Light microscopy images were examined and photographed using Zeiss Standard ICS Transmitted Light Microscope equipped with 10X and 40X CP-Achromat objective lenses. A Sony cybershot camera attached to the microscope was used to obtain images.

3.10. Statistical Analysis

Statistical analysis was done using Student’s t test program. It was used to analyze and compare the amount of food intake, serum iron levels, in control and experimental groups. Data was expressed as means with ± SEM. A p value of < 0.05 was considered statistically significant.
CHAPTER 4

RESULTS

4.1. Observations of clinical signs and behavior of the animals

Early on the experimental mice were active and consumed food and water. Water and food intake was recorded for 48 hours after 14 days. The experimental group of mice consumed 14% of their food whereas the control group consumed 41% of their food. Results indicated a significant decrease in food consumption in experimental animals. This sudden decrease in food intake may be attributed to iron overload since the control group did not show the same results. Also, after 10 days of injections, the experimental group was very aggressive during the injections as compared to the control group. However, iron injected mice did not show any signs of paralysis or unusual movements (Table 1).

4.2. Observations of clinical signs

Typically, in this project, we used the EAE scoring system from 0-5 scale. Iron overload mice did not show any signs of paralysis or weakness of the limbs (Adopted from Hooke Laboratories protocols) (Selvaraj et al. 2009). Our animal model had a score of 0 during the whole period of the experiment. This presented in detail in (Table 2).
4.3. Observations Post-Euthanasia

The abdominal cavity smelled foul. This finding was not observed in the control group of mice kept under identical conditions but sham injected with PBS. Observations were suggestive of physical and macroscopic changes due to iron overload. The organs were discolored and frail.

4.4. Serum Iron Analysis

In order to verify the success of proper injections to induce iron overload, serum concentration of iron was measured from samples taken from both sets of mice. In the experimental group, the level of serum iron was significantly elevated in comparison to control samples (p < 0.005) confirming the iron overload status (Figure 2) (Table 3).

4.5. Histology

Histological evaluation was performed on mice brain tissues from each control and experimental group. Mouse brain atlas was used to determine the brain regions. Sections stained with H&E showed infiltration and generation of inflammatory exudates around the blood vessels as described in Figure 4.

Prussian blue stain was conducted and iron deposition was found in experimental brain tissue. A quantitative analysis was performed on the iron deposition by using Image Pro software. Total blue analysis was not significant as compared to controls p-value of
0.56. Prussian blue was localized in the region of the cortex in 16 slices from 2 experimental and 2 control mice brain tissues. We analyzed 27 pictures from these slices by the software (Figure 3) (Tables 4 and 5). We observed Prussian blue stain positive in 3 slices out of 16 (19%). Data presented in the above mentioned tables.

4.6. Confocal microscopy

Longitudinal spinal cord tissues have been examined for any demyelination. Since we are using transgenic mice, the axon appears green in color and the myelin sheath appears red because of fluromyelin. Ten axons from each tissue of the control group have been examined, the myelin sheath and axons were obviously clear. The same amounts of axons have been examined for the experimental group, the myelin sheath and axons were seen very clearly. Also, we tried to find any gaps between the myelin sheath. No gaps were observed in myelin sheath surrounding the axons (Figure 5 and 6).
CHAPTER 5
DISCUSSION AND CONCLUSIONS

In this study we looked at the effect of iron overload on the myelin sheath in the CNS. Microscopic examination shows cellular infiltration in the cortex region of the brain. This infiltration may be due to inflammatory exudates of neutrophils and mononuclear cells initiated by iron overload. These findings can be supported by the flow cytometry data from the similar mice in our lab that showed significantly high levels of CD3+ T-cells with no difference in CD4+ and CD8+ T-cell ratio in the plasma of iron overload mice as compared to controls. Inflammation is a protective mechanism and one of its features is that the permeability of endothelial blood vessels increases, which explains why these inflammatory cells were observed in the brain. This data is commensurate with the studies of Experimental Autoimmune Encephalomyelitis (EAE) (Dasgupta et al. 2003).

Previous studies have shown that iron has a crucial role in the development of the EAE animal model. Mice deficient in iron did not develop EAE, whereas iron overloaded mice did develop EAE signs and symptoms (Grant et al. 2003). So, there is a link between iron overload and demyelination. In this study, unmyelinated axons have not been recognized. This data correlates with our observations, since the mice did not show any signs of paralysis or unusual movements. This study was conducted in a short period
of time, but we believe that if we had stopped the iron injections after four weeks and continued observing the mice for longer period of time 2-3 months, we may have observed demyelination. Also, since we are using male mice, iron once taken up into the tissue would not be excreted. This conclusion is supported by the fact that majority of neurodegenerative disease progress very slowly and sometimes without any symptoms.

It is worth mentioning that the behavior of the experimental group mice changed dramatically. After 10 days of injections, the experimental group was very aggressive during the injections compared to the control group. In addition, behavioral changes in food and water intake were observed; both groups were given the same amount of food and water. The diet intake was recorded for 48 hours after 14 days, and the food intake was significantly decreased in the experimental groups other than the control group. Keeping in mind that both groups were under the same stress environment, it could be attributed to iron which may inhibit the appetite of the mice.

The harmful effect of iron on mice is shown in skeletal muscle; iron has decreased the exercise performance of the skeletal muscle and increased the oxidative stress (Reardon and Allen 1994). So, if iron has a negative impact on the skeletal muscles in mice, it suggests that iron has a harmful effect on the Central Nervous System (CNS) and this suggestion was supported by our findings of iron deposits in the brain. Even though, brain is highly protected by the BBB, iron deposits have been detected which means that the permeability of the BBB is disturbed.
More recent studies have shown that iron overload leads to iron deposits in the brain (Elseweidy and Abd El-Baky 2008), so, it was believed that iron can cross the blood brain barrier. In our study, the same iron injection regime was adopted from those studies that showed that iron injection elevated the iron content of the skeletal muscle; to be sure that we will reach the iron overload condition in our mice. The serum iron level test confirmed that high serum iron levels were present in the experimental group.

The brain histological slides showed iron deposits only less than 19% in the Septo-diencephalic brain regions (S1FL) which supports the theory that iron overload makes the BBB leaky so that iron was able to escape from the brain and deposit in the brain. In Elseweidy’ study, plexus of ventricles were dilated and degenerated neurons have been seen in the cerebral cortex. This deposition in chronic conditions leads to increases in the oxidative stress and decreases in the dopamine levels (Elseweidy and Abd El-Baky 2008).

No study has investigated the effect of iron on the myelin sheath of the spinal cord because it is difficult to see any damage of myelin sheath in this region. In our study, transgenic mice were used and the axons of the CNS were brightly fluorescent all the way to the terminals of the mice spinal cord. The myelin sheath conditions were compared by the amount of fluorescence that was seen under the confocal microscope.

In the study conducted by Elsweidy, rats were fed with biscuits enriched with iron for 10 weeks and they discovered iron deposits in the brain. This contrasts with our study where we used the injection method to bypass the intestinal factor for only 4 weeks. Another point worth mentioning is that the mice which were used were male mice. The
reason of not using female mice is that hormone changes might interfere with the normal response of iron overload and some iron might be lost. If we establish the relationship between iron overload and demyelination can be established, then another study with the same procedure using female mice is strongly recommended. Research showed that estrogen plays a crucial role in the inflammatory response and there are some immunity genes that have an estrogen response element (Hewagama et al. 2008). To avoid all of these unplanned responses, we used male mice.

The oxidative stress in this project was not measured due to technical issues but research has shown that iron acts as a catalyst of reactions that lead to free radical formation and these free radicals lead to pathological changes in the body such as including decreased ATP production, oxidative damage, abnormal calcium sequestration, and apoptosis (Gordeuk et al. 1987). Therefore, in our study we concluded that there is an evidence of inflammation but no demyelination.

5.1. Limitations of this study

1. The iron levels which were used in this project do not reflect the iron levels in healthy people, but were similar to the levels that have been seen in patients with iron overload conditions.

2. Transcardinal perfusion had limited our ability to draw enough blood because we used the cardiac puncture technique.
3. Availability of more mice can solve the above limitation and allow us to do further analysis of the serum samples. Also, increase in the number of mice will make data more obvious and conclusive.

5.2. Future Study

In our study, iron is expected to increase the oxidative stress and this stress will cause damage to the oligodendrocyte cells which are responsible of demyelination (Kotze et al. 2009). So, for future research it would be useful to measure brain serotonin and dopamine levels because they are considered oxidative stress markers and have been implicated in some behavioral alterations. The same procedure could be repeated using large animals like rats and guinea-pigs. Also, acquiring blood samples during the experiment prior to transcardinal perfusion would allow for more accurate measurements.

Finally, a secondary marker could be used to confirm the nature of the infiltration to identify specific cell types. In addition, long term observation of iron overloaded mice may be needed in order to observe EAE like systems.
CHAPTER 6

TABLES AND FIGURES
Table 1: Food and water intake during 48 hour observation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Food &amp; water weight before 48 hours observation</th>
<th>Food &amp; water weight after 48 hours observation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Food</td>
<td>Water</td>
</tr>
<tr>
<td>Control</td>
<td>162.77 gm</td>
<td>250 ml</td>
</tr>
<tr>
<td>Experimental</td>
<td>108.63 gm</td>
<td>525 ml</td>
</tr>
</tbody>
</table>

Table 2: EAE scoring system for mice model. (Selvaraj et al. 2009)

<table>
<thead>
<tr>
<th>Score</th>
<th>Clinical observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No obvious changes in motor functions of the mouse in comparison to non-immunized mice.</td>
</tr>
<tr>
<td>1</td>
<td>Limp tail</td>
</tr>
<tr>
<td>2</td>
<td>Limp tail and weakness of hind legs.</td>
</tr>
<tr>
<td>3</td>
<td>Limp tail and complete paralysis of hind legs (most common).</td>
</tr>
<tr>
<td>4</td>
<td>Limp tail, complete hind leg and partial front leg paralysis.</td>
</tr>
<tr>
<td>5</td>
<td>Complete hind and complete front leg paralysis, no movement around the cage.</td>
</tr>
</tbody>
</table>
Table 3: Serum iron levels. Serum iron levels expressed as Mean ± SD. P=0.0005

<table>
<thead>
<tr>
<th>Sample</th>
<th>Serum iron levels μg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>154 ±19.0</td>
</tr>
<tr>
<td>Experimental</td>
<td>170 ±0.0</td>
</tr>
</tbody>
</table>

Table 4: Prussian blue stain analyses for the Control group.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Stain Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>31.13 ± SEM 20.96</td>
</tr>
<tr>
<td>Control 2</td>
<td>31.95 ± SEM 21.5</td>
</tr>
<tr>
<td>Control 3</td>
<td>32.13 ± SEM 21.71</td>
</tr>
</tbody>
</table>

Table 5: Prussian blue stain analyses for the experimental group.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Stain Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental 1</td>
<td>41.14 ± SEM 26.04. P value &gt; 0.05</td>
</tr>
<tr>
<td>Experimental 2</td>
<td>30.63 ± SEM 19.53. P value &gt; 0.05</td>
</tr>
<tr>
<td>Experimental 3</td>
<td>31.96 ± SEM 20.9. P value &gt; 0.05</td>
</tr>
</tbody>
</table>

In Tables 4 and 5, all data are expressed as Mean ± SEM. P < 0.05 is considered significant.
Figure 1: Generation of reactive and damaging hydroxyl radicals (OH•) (modified from article by Khalil et al. 2011). Free Iron (Fe2+) reacts through the Fenton reaction with hydrogen peroxide, leading to the generation of very reactive and damaging hydroxyl radicals (OH•). Superoxide can also react with ferric iron in the Haber-Weiss reaction leading to the production of Fe2+, which then again affects redox cycling. The highly reactive hydroxyl radicals lead to oxidative stress-induced lipid peroxidation, mitochondrial dysfunction, and increase in intracellular free-calcium concentration, and finally causing neuronal death (Khalil et al. 2011).
Figure 2. Serum iron levels: Graph shows serum iron level in control and experimental groups of mice. The serum iron level is significantly different from the control group *(P<0.05).*
Figure 3: Prussian blue stained Control and experimental in cortex of

*Septo-diencephalic brain regions (S1FL).* Mice were injected with iron dextran by intraperitoneal injection (IP) for 5 days for 4 weeks. Blue arrow shows iron deposition. Digital images were collected under bright-field setting using a 40x objective by light microscopy.
Figure 4: Hematoxylin and eosin (H&E)-stained control and experimental sections from the cortex of Septo-diencephalic brain regions (VMH): Hematoxylin and eosin (H&E)-stained cortical region of control on the left and experimental on the right brain section. Blue arrow shows Perivascular Cuffing indicating mononuclear cells around blood vessels. Control mice were injected with PBS and experimental mice with Iron dextran intraperitonealy for 5 days for 4 weeks. Digital images were collected under bright-field setting using a 40 x objective by light microscopy.
Figure 5: Spinal cord tissue of control mouse stained with fluromyelin stain.
Longitudinal Spinal cord section from control mice were stained with fluromyelin stain. Dorsal column spinal cord axon appears as green color. Myelin sheath appears as red color imaged by confocal microscopy, equipped with 63X plain Apochromatic Objective.
Figure 6: Spinal cord tissue of experimental mouse stained with fluromyelin stain.

Longitudinal Spinal cord sections from experimental mice were stained with fluromyelin stain. Dorsal column spinal cord axon appears as green color. Myelin sheath appears as red color imaged by confocal microscopy, equipped with 63X plain Apochromatic Objective.
References


