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Human, animal, and cell culture studies support the relationship between iron overload and lowered bone mineral density. The hypothesis of the studies in this thesis was that: iron accumulates intracellularly and that this accumulation results in increased iron levels that alter iron-regulatory proteins and also maximally suppress osteoblast maturation and function. Osteoblast-like cells isolated from fetal rat calvaria were treated with ferrous sulfate ($FeSO_4$) (0-10 μM). Intracellular iron concentration was increased 25-fold greater than control upon 5 μM $FeSO_4$ exposure. Transferrin receptor and ferritin light-chain protein and gene expression were altered and osteoblast phenotypes were markedly suppressed with excessive $FeSO_4$ treatment. Excessive $FeSO_4$ treatment resulted in high intracellular iron accumulation, alterations in key iron-regulated gene and protein expression, and the suppression osteoblast maturation and function in a concentration-dependent and time-dependent manner. These results provide the basis for evaluation of mechanisms by which iron overload alters osteoblast maturation and function.

THE EFFECT OF IRON OVERLOAD ON OSTEOBLAST FUNCTION IN CELL CULTURE

by

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Approved by

_____
Committee Chair

To my extraordinary family in its entirety, you are my world.

Every one of you has made up who I am today.

To the Yaya's, beautiful and brilliant.

To Aether, my balance and Friend.

Thank you.

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Iron is an essential metal that is a part of many proteins utilized by the body for cellular growth and survival. Ribonucleotide reductase, the electron transport chain, and oxygen transportation all utilize proteins that are iron-containing (Beard 2001, Greene et al. 2002). Enzymes in iron-sulfur proteins participate in reduction/oxidation (redox) reactions in the electron transport chain. Aconitase, a regulatory protein, and ferrochelatase are non-redox enzymes that are iron-containing proteins (Arredondo et al. 2005). Additionally, catalases and myeloperoxidases are both iron-containing enzymes. Catalase is extremely important to the prevention of hydrogen peroxide (H_2O_2) induced cellular damage by converting it to water and molecular oxygen (Chiancone et al. 2004).

Although iron is a trace element that is pertinent to human survival, its imbalance can cause adverse damaging effects and these effects are seen through a multitude of disorders. Iron deficiency, usually from low dietary intake of iron or excessive bleeding, is the most prevalent type of iron disorder in humans (Walter et al. 2002). On the other extreme iron overload can result from genetic disorders, excessive iron supplementation, and age-related iron accumulation. Hemochromatosis, aceruloplasminemia, and

Friedreich's ataxia are some of the genetic mutations that can create iron overload in humans (Sheth et al. 2000). Parenteral iron, iron via intravenous feeding, and iron supplementation are other examples of how iron levels in the body can accumulate without a physiological disposition to iron overload (Fischer et al. 2004, Blanck 2005, Puntarulo 2005, Besarab et al. 1999). Dietary iron overload, from heme iron and fortification, in healthy humans has also been shown to contribute to iron overload (Liu et al. 2003, Mainous et al. 2004, Milman et al. 2003). Iron overload, in physiological conditions like hemochromatosis, has been linked to decreased insulin sensitivity and increased risk of diabetes (Jehn et al. 2004).

Recently, hemochromatosis has been linked to decreased bone mineral density (BMD) and increased risk of osteoporosis in humans (Guggenbuhl et al. 2005, Anelopoulos 2006). *In vitro*, osteoblast cells express the iron-regulated proteins transferrin receptor and ferritin throughout differentiation, suggesting that iron is important in osteoblast maturation and function (Gentili et al. 1994, Kasai et al. 1990, Spanner et al. 1995). Initial studies in our lab have identified that excessive treatment doses of ferrous sulfate ($FeSO_4$) markedly inhibits osteoblast maturation and function *in vitro* (Kilbarger et al., unpublished). Formation of alkaline phosphatase-positive colonies and multi-layered mineralized nodules were markedly suppressed with excessive iron treatment in osteoblast-like cells isolated from fetal rat calvaria. Additionally, genes characteristic of development of the osteoblast phenotype, alkaline phosphatase, bone sialoprotein and osteocalcin, were also dramatically suppressed. These preliminary results suggest that excessive iron concentrations may inhibit development of the

osteoblast phenotype *in vitro* and consequently impair bone formation. However, the extent to which intracellular iron concentration is related to osteoblast function and alterations in iron-regulatory proteins is unknown. Thus, the overall hypothesis is that: iron accumulates intracellularly and that this accumulation results in increased iron levels that alter iron-regulatory proteins and also maximally suppress osteoblast maturation and function. The specific aims of this project were to determine the concentration-dependent and time-dependent relationship between excessive iron treatment and 1) intracellular iron concentration, 2) gene and protein expression of iron-regulatory protein, and 3) development of osteoblast phenotype using osteoblast like cells isolated from primary fetal rat calvaria. These results provide the basis on which specific mechanisms of iron overload on osteoblast maturation and function can be evaluated.

CHAPTER II

REVIEW OF PERTINENT LITERATURE

Bone remodeling is a sensitive balance between osteoclasts and osteoblasts. Osteoclasts originate from mononuclear hematopoietic precursors and become multinucleated cells that are responsible for bone resorption. Osteoclasts commence bone turnover by dissolving mineralized bone making a lacunae. This process then ceases and osteoblasts, derived from mesenchymal cells, begin rebuilding the area created by the osteoclasts by secreting bone matrix which is then mineralized. Cell secretion of alkaline phosphatase and different noncollagenous proteins such as osteocalcin and bone sialoprotein are characteristic of mature osteoblast phenotypes (Aubin 1998, Voskaridou et al. 2004, Hinoi et al. 2006). Additionally, osteoblasts express Ligand to receptor activator of NFκB (RANK-L), a key regulator of osteoclast activation. Imbalances in bone deposition and resorption can result in conditions such as osteoporosis, osteopetrosis, and osteomalacia. A reduction in BMD can lead to osteoporosis and is a result from an overproduction of osteoclast number or an underproduction of osteoblast number. Osteopetrosis, excessive bone mass, is due to increased bone formation or decreased bone resorption (Cohen 2006). Osteomalacia is a result of impaired mineralization that leads to excessive build up of osteoid tissue. Thus,

metabolic bone diseases can result from imbalances in osteoblast and/or osteoclast production (Cohen 2006).

Osteoblast cells express the iron-regulatory proteins transferrin receptor and ferritin during differentiation in vitro, suggesting a role of iron in osteoblast function (Gentili et al. 1994, Kasai et al. 1990, Spanner et al. 1995). Transferrin receptor and ferritin are major iron-regulated proteins involved in transporting and storing iron. After dietary iron is transported across enterocytes it is then bound and transported to tissues by serum transferrin proteins. The iron bound transferrin can then be endocytosed by binding to transferrin-receptors on the cell surface. Ferritin is an iron storage protein consisting of light and heavy chain elements. Ferritin light chain is more prevalent in tissues that require long term storage, such as the spleen and liver. Heavy chain ferritin levels are higher in tissues that require a rapid need for iron, such as the heart. In osteoblasts ferritin light has been shown to be more prevalent in comparison to ferritin heavy (Spanner et al. 1995). Iron regulatory proteins (IRP) regulate iron balance by binding to iron regulatory elements (IRE) located on mRNA sequences, resulting in the regulation of protein translation. When intracellular iron concentration is low, IRPs bind to IREs and transferrin receptor mRNA stability and synthesis is increased. At the same time the translation of ferritin mRNA into proteins is repressed. In contrast, when intracellular iron concentration is high the IRPs are converted to cytosolic aconitase or degraded and transferrin receptor proteins are degraded and mRNA stability is decreased, while ferritin mRNA translation is activated (Crichton et al. 2002, Eisenstein 2000).

Under normal conditions these processes help to regulate intracellular iron concentration levels when deficient or excessive to protect cellular survival.

Growth factors, such as insulin growth factor-1 (IGF-1), epidermal growth factor (EGF), and platelet-derived growth factor have been reported to regulate transferrin and iron uptake in human epidermoid carcinoma cells and multiple fibroblast lines. Both IGF-1 and EGF increased the cycling, both endocytosis and exocytosis, of diferric transferrin bound transferrin receptor (Davis et al. 1986, Davis et al. 1987). Growth factors were reported by Davis et al. (1986) in rat adipocytes to increase the uptake and accumulation of iron through transferrin receptor endocytosis. In addition, these authors reported strong associations between cellular growth and transferrin receptor. Mahachoklertwattana et al. (2003) reported a correlation of reduced bone maturation with heightened iron deposits and insulin growth factor I (IGF-I). Although these findings have not been reported in osteoblasts, it is possible that differentiating preosteoblasts could potentially be influenced by growth factors and their effects on iron uptake.

Although iron is a trace element that is required for human survival, its imbalance can cause adverse damaging effects and these effects are seen through a multitude of disorders. Iron deficiency, usually from low iron dietary intake or excessive bleeding, is the most prevalent type of iron disorder in humans (Walter et al. 2002). On the other extreme iron overload can result from genetic disorders, excessive iron supplementation, and age-related iron accumulation. Hemochromatosis, aceruloplasminemia, and Friedreich's ataxia are some of the genetic mutations that can create iron overload in humans (Sheth et al. 2000). Parenteral iron, iron via intravenous feeding, and iron

supplementation are other examples of how iron levels in the body can accumulate without a physiological disposition to iron overload (Fischer et al. 2004, Blanck 2005, Puntarulo 2005, Besarab et al. 1999). Dietary iron overload, from heme iron and fortification, and excessive iron supplementation in healthy humans has also been shown to contribute to iron overload (Blanck et al. 2005, Fisher et al. 2004, Liu et al. 2003, Mainous et a. 2004, Milman et al. 2003). Accumulation of stored iron, measured by ferritin levels, has also been shown to increase with aging (Johnson et al. 1994, Fleming et al. 2001).

Iron is also a cofactor for key enzymes, such as prolyl and lysyl hydroxylases, involved in collagen synthesis (Ilich et al. 2000, Medeiros et al. 2004). Collagen is the primary protein in the bone matrix, thus iron status may impact bone mass (Cohen 2006). Iron intake in post-menopausal women and serum ferritin levels in young pre-menopausal women have also been found to have a positive correlation to BMD (Ilich et al. 2000, Maurer et al. 2005). Iron deficiency in young, growing rats lowers bone mass and increases bone fragility, thus the skeleton is impacted by iron levels (Medeiros et al. 2002, Medeiros et al. 2004). Dietary iron deficient and hypotransferrinemic mice were reported to have lower bone mineralization in a study conducted by Malecki et al. (2000). Thus, iron is a requirement for normal bone mineralization.

Iron Overload and the Skeleton

An association of reduced bone mass with iron overload in humans has been reported. For example, osteopenia and osteoporosis have both become recognized as conditions that commonly occur in individuals with iron overload from genetic

conditions such as hemochromatosis (Weinberg et al. 2006). Hemochromatosis has also been linked to lower BMD and increased risk of osteoporosis in men (Diamond et al. 1989, Guggenbuhl et al. 2005, Angelopoulos 2006). Guggenbuhl et al. (2005) evaluated BMD using dual-energy X-ray absorptiometry at the lumbar spine and femoral neck region in thirty-eight men, mean age of 47 years, with genetic hemochromatosis (GH). Of the men studied, 78.9% had osteopenia and 34.2% had osteoporosis. These results were similar to another study (Sinigaglia et al. 1997) conducted on GH males that also used dual-energy X-ray absorptiometry. Higher hepatic iron levels in this study were inversely correlated with lower femoral neck BMD. Mahachoklertwattana et al. (2003) also reported decreased BMD in the lumbar spine of patients with hereditary β–thalassemia/ hemoglobinopathy who had iron overload from blood transfusions. These patients also had decreased bone matrix maturation and mineralization, determined using bone histomorphometry. The authors concluded there was a correlation of reduced bone maturation with heightened iron deposits in bone and low serum levels of insulin growth factor I. Although results of these human studies suggest an inverse correlation between iron overload and lower BMD, the extent that excess iron alters osteoblast or osteoclast development and function is unclear.

Some insights into cellular targets of iron overload have been reported using animal models. Vernejoul et al. (1984) found that iron overloaded pigs had significantly low bone formation and a lower number of osteoblasts, in the presence of normal osteoclast function and normal bone resorption. In the study of Matsushima et al. (2001), male Sprague-Dawley rats fed 50,000 ppm (5%) iron lactate had significantly lower body

weights compared to the control pair-fed group. Iron lactate fed rats had significantly lower bone volume, trabecular number, and trabecular thickness than the pair-fed control group. Bone resorption and bone formation were both higher at 4 weeks of iron lactate overload, along with reductions of alkaline phosphatase (30%) and inorganic phosphorous (14%) in comparison to 2 weeks of iron lactate overload. Urinary calcium and iron levels were both significantly higher in both iron overloaded rats in comparison to control. Tartrate-resistant acid phosphatase, an osteoclast resorption enzyme, was also stimulated by iron lactate overload**.** Thus, the existing literature supports the hypothesis that both osteoblasts and osteoclasts may be impacted by iron overload.

Mechanisms by which iron overload alters the skeleton are unknown. However, animal studies also showed that oxidative stress from iron overload may contribute to the development of metabolic bone disease. Isomura et al. (2004) evaluated reactive oxygen species (ROS) and bone metabolism in postmenopausal Wistar rats with iron overload, induced by a high (5%) iron lactate diet. Dietary iron overloaded postmenopausal rats had significantly lower body weights in comparison to controls, one postmenopausal control-diet group and one young control-diet group. Serum levels of 8-hydroxy-2'-deoxyguanosine, a marker of DNA oxidative damage, was significantly greater in dietary iron overloaded postmenopausal rats and young control diet rats in comparison to postmenopausal control diet rats, the reason for greater levels in young control rats was unknown. Serum levels of glutathione peroxidase, an antioxidant released by osteoblasts in response to ROS, was dramatically lower in dietary iron overloaded postmenopausal rats in comparison to both controls. Tumor growth factor –β (TGF-β), a cytokine found

to alter RANK-L, was 30-fold greater in urine and almost 2-fold greater in serum analysis. TGF-β levels were found to be negatively correlated to glutathione peroxidase levels in iron fed rats in comparison to controls. In another study (Liu et al. 2006), postmenopausal female rats were ovariectomized (OVX) to create estrogen deficiency inducing peri-/ postmenopausal osteoporosis and were then compared to non OVX rats. The OVX groups had a significantly greater level of free iron in cortical bone, determined by electron paramagnetic resonance spectrometry, in comparison to control groups. 1-N-docosyl-triethylenetetraminepentaacetic acid, a bone targeting chelator that has a high iron binding affinity, was given to some OVX rats for nine weeks. The binding of iron by chelation resulted in less bone loss due to OVX. Cancellous BMD was higher in chelator-treated rats in comparison to control OVX rats. Histomorphometric data showed a significantly lower cancellous bone resorption and trabecular separation, along with greater trabecular bone mass in chelator-treated OVX rats compared to non treated OVX rats. Iron chelation reduced estrogen deficient induced bone loss, suggesting that high iron levels accumulated in bone may contribute to post-menopausal bone loss. Thus, ROS or other chemical changes induced by iron accumulation contribute to bone loss.

Oxidative Stress and the Skeleton

Although the mechanism(s) responsible for bone loss with iron overload are unknown, the generation of ROS is a likely contributor. ROS are considered a contributor in the development of osteoporosis, independent of iron status. In a cross-sectional study by Maggio et al. (2003), antioxidant levels in plasma were shown to be

dramatically lower in osteoporotic women in comparison to controls. Studies have also reported a positive relationship between increasing dietary antioxidant intake and BMD (Hall et al. 1998, Leveille et al.1997). Oxidative stress was shown to have a negative affect on BMD in a study by Basu et al. (2001). This study measured BMD and 8-Iso-$PGF_{2\alpha}$, an oxidative marker, in 48 women and 53 men with a mean age of 55.8 years. Lower BMD was shown to be significantly related to elevated 8-Iso-$PGF_{2\alpha}$. The measurement of an inflammatory marker, 15-keto-dihydro-$PGF_{2\alpha}$, also showed negative effects on BMD. Oxidative stress has been shown overall to have negative correlation to BMD.

Evidence *in vitro* also suggests that ROS alter osteoblast function. Arai et al. (2007) reported that H_2O_2-treated MC3T3-E1 osteoblastic had a marked decreased in nodule formation, differentiation, and osteogenic marker gene expression of alkaline phosphatase, bone sialoprotein, and runt-related transcription factor 2 in comparison to non-treated cells. Hinoi et al. (2006) reported that Nrf2 transfected MC3T3-E1 osteoblastic cells altered runt-related transcription factor 2 transcription, while also lowering osteoblast differentiation and alkaline phosphatase activity in comparison to non-transfected cells. A further study by Bai et al. (2004) evaluated that oxidative stress stimulated the extracellular signal-regulated kinases and nuclear factor-κB signaling pathways using primary rabbit calvarial osteoblast and bone marrow stromal cells treated with H_2O_2. This study also reported that treated cells had lower alkaline phosphatase staining and osteoblast colony forming units in comparison to non-treated cells.

Oxidative stress was reported in these studies to inhibit osteoblast function and maturation.

Cellular death, by apoptosis or necrosis, is one proposed mechanism by which iron imbalance or overload can damage cells and reduce proper cellular development and function. Evidence exist that iron overload induces cell death by apoptosis and necrosis by generation of ROS (Rauen et al. 2004). Iron is a potent pro-oxidant and its reducing capabilities make it an instigator for increasing ROS (Crichton et al. 2002). High levels of iron or free ferrous iron can react with H_2O_2 initiating the Fenton reaction (Tenopoulou et al. 2005). The end product of this reaction includes unstable free radicals and oxidized ferric iron. Iron is a main transition metal that catalyzes the Haber Weiss reaction, a secondary reaction to the Fenton reaction (Puntarulo 2005, Simunek et al. 2005). A superoxide radical (O^{2-}) generated from the Fenton reaction reacts with H_2O_2, which in turn creates molecular oxygen and free hydroxyl radicals. One specific hydroxyl radical that can be generated is ·OH which can induce oxidative damage to cell membranes, lipids, and even direct damage on DNA (Crichton et al. 2002). Oxidative stress can initiate apoptosis or necrosis in cells depending on the concentration of free radicals, H_2O_2 at very high levels results in necrosis of cells (Hampton et al.1998).

Doulias et al. (2003) and Tenopoulou et al. (2005) found that iron toxicity, in HeLa and Jurket cells, significantly reduced cell proliferation in comparison to controls. Increased intracellular iron, in comparison to extracellular iron, has been shown in Rauen et al. (2004) to have a stronger effect on cellular damage. In this study the treatment of rat hepatocytes with iron, Fe(III)/8-hydroxyquinoline, induced precursor signs of

apoptosis and necrosis. Most of the cellular injury from the iron treatment showed early signs of apoptosis, such as chromatin condensation, increased mitochondrial permeability, and nuclear shrinkage. A smaller portion had swollen nuclei and blebbing showing the initiation of cell death by necrosis. Barbouti et al. (2001) had similar findings, utilizing Jurket cells, that intracellular redox-active iron takes part in DNA damage along with H_2O_2. Desferrioxiamine, a synthetic iron chelator, has also been reported to lower apoptosis and nuclear DNA damage inflicted by oxidative stress (Barbouti et al. 2001, Doulias et al. 2003, Kurz et al. 2004, Tenopoulou et al. 2005).

Human, animal, and cell culture studies support the relationship between iron overload and lowered BMD. *In vitro* and *in vivo* studies also suggest that one possible mechanism is through ROS. However, little is known about the relationship between excess iron, intracellular iron accumulation in osteoblast, and osteoblast maturation and function. The specific aims of this project were therefore, to determine the concentration-dependent and time-dependent relationship between excessive iron treatment and 1) intracellular iron concentration, 2) gene and protein expression of iron-regulatory proteins, and 3) development of osteoblast phenotype using osteoblast-like cells isolated from primary fetal rat calvaria. These results provide the basis on which specific mechanisms of iron overload on osteoblast maturation and function can be evaluated.

CHAPTER III

THE EFFECT OF IRON OVERLOAD ON OSTEOBLAST FUNCTION IN CELL CULTURE

Abstract

Although iron overload has a negative impact on BMD *in vivo*, the impact of iron overload on osteoblast function and maturation has not been reported. This study investigates the extent that iron accumulates intracellularly in osteoblasts and if this accumulation is associated with altered iron-regulatory proteins and suppressed osteoblast maturation and functions. Osteoblast-like cells isolated from fetal rat calvaria were treated with 0-10 μM ferrous sulfate ($FeSO_4$). Intracellular iron concentration was increased 25-fold greater than control with 5 μM $FeSO_4$ exposure. As expected, excessive $FeSO_4$ treatment doses decreased both transferrin receptor gene and protein expression, while ferritin light-chain protein expression was increased. Osteoblast function, shown by percent mineralized surface area, and osteoblast maturation, shown by osteocalcin, bone sialoprotein, and alkaline phosphatase gene expression, were markedly suppressed with excessive $FeSO_4$ treatment doses. Excessive $FeSO_4$ treatment results in high intracellular iron accumulation, alterations in key iron-regulated gene and protein expression, and the suppression osteoblast maturation and function. These results provide the basis for evaluation of mechanisms by which iron overload alters osteoblast maturation and function.

Introduction

Iron is a trace element that is pertinent to human survival, and an imbalance in iron status may result in metabolic dysfunction. For example, iron overload can contribute to conditions such as atherosclerosis, cancer, diabetes, and dementia (Liu et al. 2006). Iron overload may result from genetic disorders, excessive iron supplementation, and age-related iron accumulation. Hemochromatosis, aceruloplasminemia, and Friedreich's ataxia are some of the genetic mutations that can result in iron overload in humans (Sheth et al. 2000). Dietary iron overload, from heme iron and fortification, and excessive iron supplementation are other examples of how iron levels in the body can accumulate without a physiological disposition to iron overload (Fischer et al. 2004, Blanck 2005, Puntarulo 2005, Besarab et al. 1999, Liu et al. 2003, Mainous et a. 2004, Milman et al). Accumulation of stored iron, measured by ferritin levels, has also been shown to increase with aging (Johnson et al. 1994, Fleming et al. 2001).

Osteoporosis, osteopetrosis, and osteomalacia are examples of metabolic bone diseases that can result from imbalances in osteoblast and/or osteoclast production or function (Cohen 2006). These conditions have been reported in individuals with iron overload (Weinberg et al. 2006, Diamond et al. 1989, Guggenbuhl et al. 2005, Angelopoulos 2006). Iron overload was inversely correlated with BMD in genetic disorders such as hemochromatosis (Guggenbuhl et al. 2005, Anelopoulos 2006). Oxidative stress from iron overload contributes to the development of metabolic bone disease in animal studies (Isomura et al. 2004). Vernejoul et al. (1984) found that iron overloaded pigs had significantly low bone formation and a lower number of osteoblasts,

in the presence of normal osteoclast function and normal bone resorption. This suggests that iron overload may alter osteoblast function.

The primary goal of our study was to determine if iron accumulates intracellularly and how this accumulation alters iron-regulatory proteins and potentially suppresses osteoblast maturation and function. To investigate these effects, we analyzed the concentration-dependent and time-dependent relationship between excessive iron treatment and 1) intracellular iron concentration, 2) gene and protein expression of iron-regulatory proteins, and 3) the development of osteoblast phenotype using osteoblast-like cells isolated from primary fetal rat calvaria.

Methods

Cell Culture: Time-pregnant Sprague-Dawley rats were obtained on day 13 of pregnancy (Harlan, SD, Raleigh, NC) and housed at 19-20ºC with a 12h light-dark cycle. Rats were provided Harlan Teklad 7002 6% mouse/rat diet and water ad libitum. Dams were sacrificed at day 21 of pregnancy by CO_2 overdose and pups were removed. These procedures were approved by the Institutional Animal Care and Use Committee (IACUC).

Calvaria were aseptically removed, cleaned of residual tissue, and digested using five sequential collagenase digestions. Cells were allowed to attach and incubate for 24 hours in α-Modified Eagle's Medium (FBS), 15% Fetal Bovine Serum, 10% antibiotics. Cells were trypsinized and then seeded at 3,000 cells/cm² in 6 well plates. The cells were grown in osteogenic culture media, containing α-Modified Eagle's Medium, 10% Fetal Bovine Serum, 10% antibiotics, 1% ascorbic acid, 1% sodium β-glycerolphosphate, and 10^{-8} dexamethasone. Cells were held in a 37ºC incubator with 95% air and 5% CO_2.

At confluence, approximately day 8 of culture, treatment with Iron(II) sulfate heptahydrate $FeSO_4$ (Sigma, F7002-250G) (1-10 μM $FeSO_4$) began. Fresh media and $FeSO_4$ were provided every 2-3 days. Fresh $FeSO_4$ stock solution was prepared each time by dissolving $FeSO_4$ in deionized water and serially diluted in media before media changes. Final concentrations range from 0-10 μM $FeSO_4$. Deionized water was utilized as the control (0 μM) treatment. Iron concentration values were confirmed by Graphite Furnace Atomic Absorption Spectrometry (See Appendix). The 0 μM dose contained 0.003 μmol/L iron, due primarily to the iron contained in FBS. Percent increase above

control (0 μM) in $FeSO_4$ treatment doses are as follows: 1 μM (32% increase), 2 μM (61% increase), 3 μM (86% increase), 4 μM (118% increase), and 5 μM (134% increase). Some studies also used a 10 μM dose, but this dose was not analyzed spectrometrically.

Graphite Furnace Atomic Absorption Spectrometry (GFAAS): GFAAS was used to measure the iron concentration in reagents and also intracellular iron concentrations using a protocol adapted from Erikson et al. 2006. At the midpoint (D15) and end of culture (~D20 – D21), media was removed, and cells were washed twice with PBS. Cells were scraped off the cell culture plate in PBS**,** pipeted into 1.5mL microcentrifuge tube, then digested in 100% Ultra Pure Nitric Acid at 60ºC for 48 hours in a sand bath in a fume hood. A 1:20 dilution of digested cell lysate in ultra pure nitric acid was subjected to GFAAS. Media and reagents were also digested and analyzed as described above. Protein concentration of cell digest was assessed by bicinchoninic acid assay (BCA) (Pierce, Product #23227) and normalized values are expressed as nmol Fe/ mg protein.

Staining: At the end of culture (~ D20 – D21), cells were stained for alkaline phosphatase positive colonies (deep pink), which indicates cells that have potential to become osteoblasts. Cells were then counterstained with 2.5% silver nitrate (von Kossa stain), which stains mineralized nodules dark brown. Plates were scanned and the percentage of surface that was mineralized was determined using Adobe Photoshop (version 6.0).

Semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR): Cells were scraped from wells in 1mL of TRIZOL (Invitrogen) and RNA was extracted using procedures specified by manufacturer. Isolated RNA was dissolved in 20μL of

DEPC water and DNase treated. RNA purity and concentration were determined spectrophotometrically at 260 and 280 nm using a Beckman Spectrophotometer. 1µg of RNA was reverse transcribed using 1µg Oligo dT and an Omniscript RT kit (Qiagen) by manufacturers instructions. cDNA was then be diluted to 1:10 in nuclease-free water. As a procedural control, PCR was performed on RNA not subjected to reverse transcription to confirm the absence of nonspecific interference. Reagent contamination was controlled for by using nuclease free water in place of cDNA during the reverse transcription reaction. 1µL of the diluted cDNA was used in the PCR reactions using a Taq polymerase PCR kit (Qiagen) and primers (refer to Table 1) specific for bone sialoprotein, osteocalcin, transferrin receptor, alkaline phosphatase and ribosomal L32. Primers were designed to span exon/exon junction to control for nonspecific PCR products. Each gene was optimized within the exponential phase of amplification curve. PCR products were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide. Gels were visualized with ultra-violet light and net intensities quantified using Kodak Imaging Station. Net intensities were normalized to the mean net intensity values of ribosomal L32.

Table 1: RT-PCR primer information.

Primer	Sequence	Annealing Temperature	Fragment Size
Ribosomal L32 [1]	**F-** CAT GGC TGC CCT TCGGCC TC **R-** CAT TCT CTT CGC TGC GTA GCC	56°	403bp
Osteocalcin [1]	**F-** AGG ACC CTC TCT CTG CTC AC **R-** AAC GGT GGT GCC ATA GAT GC	56°	274bp
Transferrin Receptor [2]	**F-** GGC CGG TCA GTT CAT TAT TA **R-** CTC ATG ACG AAT CTG TTT GTT	55°	237bp
Bone Sialoprotein [1]	**F-** CGC CTA CTT TTA TCC TCC TCT G **R-** CTG ACC CTC GTA GCC TTC ATA G	56°	780bp
Alkaline Phosphatase	**F**- GAC CTT GAA AAA TGC CCT GA **R**- CGC ATC TCA TTG TCC GAG TA	56°	474bp

[1] Bonnelye et al. 2001, [2] Liu et al. 2003

Western Blotting: Proteins were collected in 150 µL of RIPA lysis buffer, containing protease inhibitor cocktail kit (Calbiochem, Cat. #539131), 10 mM sodium fluride, 20 mM β-glycerol phosphate, 0.1 mM sodium orthovanadate. Proteins were then sonicated on ice and centrifuged (Eppendorf 5424) at 16,000 x g for 20 minutes. Supernatants were removed and stored in -80ºC freezer. Protein concentrations were determined as described above. 20µg of protein was electrophoresed in NuPage 4-12% Bis-Tris gels (Invitrogen, Cat. NPO322BOX) and proteins were transferred for 3.5 h onto polyvinylidene difluoride membrane (Immobilon, Cat. IPVH00010). The membrane was then blocked with 5% milk in Tris Buffer Saline with Tween-20 (TBS-T) (SigmaUltra, Product #P7949-500ML). The membrane was then incubated overnight at 4°C with primary antibodies in 5% Bovine Albumin (Sigma, #A-9418)/ TBS-T. Primary Abs include anti-β-actin (Sigma, #A-5441), anti-human transferrin receptor (Zymed, #13-16800), anti-ferritin rabbit (Alpha Diagnostic, #FerL14A). Secondary antibodies are

donkey anti-mouse IgG (Affinity Bioagents, #SA1-100) for β-actin and TrfR, and goat anti-rabbit IgG (Cell Signaling, #7044) for ferritin light chain. Both secondary antibodies were conjugated to horseradish peroxidase and then incubated with membrane for 30 minutes at room temperature. The membrane was washed with TBS-T and analyzed with Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer, #NEL105) per manufacturer protocol. ß-Actin was used as a loading control. ReStore Western Blotting Stripping Buffer (Pierce, #21059) was used to strip membrane per manufacturer protocol and re-probed for 30 minutes with mouse anti-actin (Sigma, #A-5441) in 5% milk/ TBS-T. It was then washed in TBS-T and probed with horseradish peroxidase -conjugated donkey anti-mouse IgG (Affinity Bioagents #SA1-100).

Statistics: One-way analysis of variance (ANOVA) was used to determine the significance of differences among treatments for RT-PCR, percent surface area mineralization, and intracellular iron concentration levels. A two-way ANOVA was used to evaluate the significance of differences in the main effects of treatment and time when variables were evaluated at two time points of culture. Where the interaction was significant, a one-way ANOVA was conducted separately for each time point. Statistical differences between treatments were determined by Tukey HSD post hoc analysis. $P < 0.05$ was considered significant.

Results

The Effects of Iron Overload on Intracellular Iron

Study 1

Intracellular iron concentrations increased in a dose-dependent manner at D21 of cell culture (Figure 1). The intracellular iron concentration of control cells (0 µM) was 2.56 +/- 0.02 nmol Fe/mg protein (n = 3 wells). There was an approximately 20 times ($p < 0.05$) higher intracellular iron level at 5 µM and 10 µM FeSO4, in comparison to the 1 µM and the control (0 µM) doses. There were no differences in intracellular iron concentration levels between the 1 µM $FeSO_4$ and the 0 µM doses. Differences in intracellular iron concentrations levels with 5 µM and 10 µM $FeSO_4$ treatments were also not significantly different.

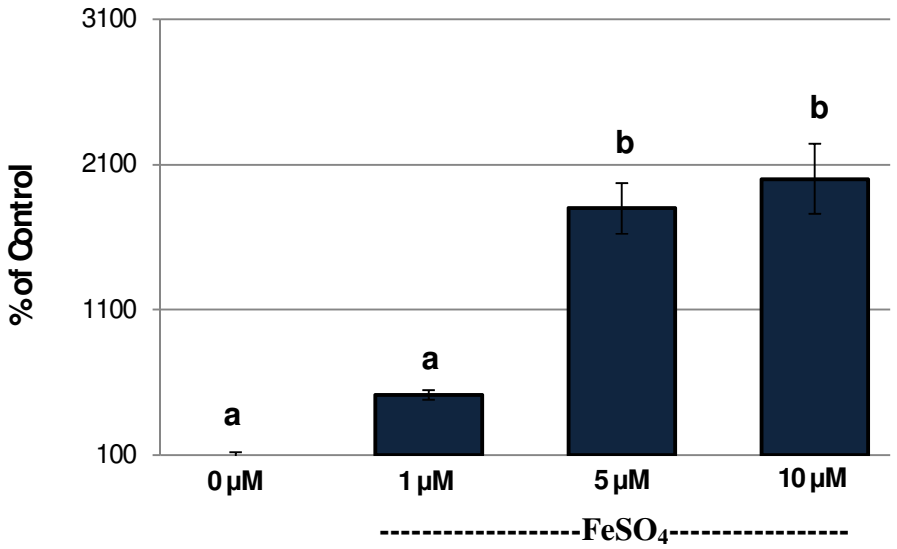

Figure 1: Intracellular iron concentration (% of control) in osteoblast-like cells isolated from fetal rat calvaria at D21 of cell culture. Results represent the mean +/- SEM, n = 3 - 5 wells analyzed per treatment for intracellular iron and n = 3 wells analyzed for protein (nmol of Fe/mg of protein) for each treatment. Mean differences, determined by Tukey HSD ($p <$ 0.05), are indicated by letters.

Study 2

Because there were no differences between the 5 µM and 10 µM $FeSO_4$ doses the effect of time of culture and treatment doses lower than 5 µM $FeSO_4$ on intracellular iron concentration were evaluated (Figure 2). At D15, control cells contained 3.05 +/- 0.14 nmol Fe/mg protein (n = 6). Intracellular iron concentration of control cells was lower on D20 of culture (1.85 +/- 0.08 nmol Fe/mg protein, n = 6) than on D15. Due to a significant time and treatment interaction, results for D15 and D20 were analyzed by separate one-way ANOVA. At D15 of cell culture, the 2 µM and 3 µM $FeSO_4$ treatment had approximately 10 times higher ($p < 0.05$) intracellular iron levels in comparison to the 0 µM (control) (Figure 2A). The 4 µM and 5 µM $FeSO_4$ doses were 25 to 30 times higher, respectively, than the 0 µM (control). The 4 µM dose was approximately 25% lower ($p < 0.05$) than the 5 µM $FeSO_4$ dose. At D20 of cell culture intracellular iron concentration levels increased with increasing treatment doses of $FeSO_4$ (Figure 2B). Intracellular iron concentration level was approximately 40 times higher at 5 µM $FeSO_4$ dose when compared to control ($p < 0.05$).

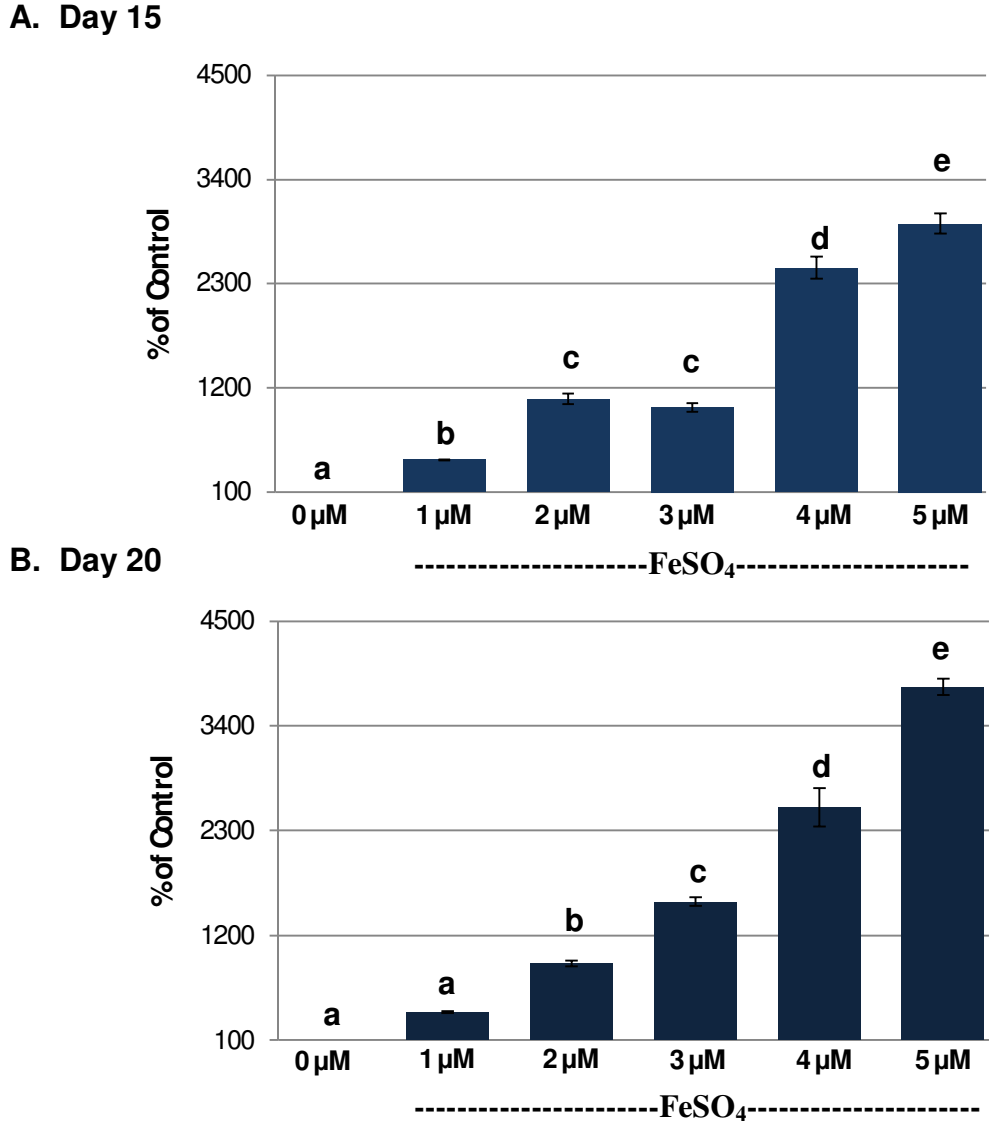

Figure 2: Intracellular iron concentration (% of control) in osteoblast-like cells isolated from fetal rat calvaria on D15 and D20 of cell culture. (A) D15 of cell culture. (B) D20 of cell culture. Results represent the mean +/- SEM, n = 6 wells analyzed per treatment for intracellular iron and n = 6 wells analyzed for protein for each treatment. Mean differences, determined by Tukey HSD ($p < 0.05$), within the day are indicated by letters.

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The Effects of Iron Overload on Iron-Regulatory Gene and Protein Expression

Study 1

Gene expression of transferrin receptor was suppressed at D21 of cell culture with excessive $FeSO_4$ treatment (Figure 3). Representative gels (Figure 3A) demonstrate that transferrin receptor was decreased markedly with 1 μM $FeSO_4$ treatment. The 5 μM and 10 μM $FeSO_4$ treatments had an even more pronounced suppressive affect in comparison to 1 μM $FeSO_4$ and 0 μM (control) (Figure 7B).

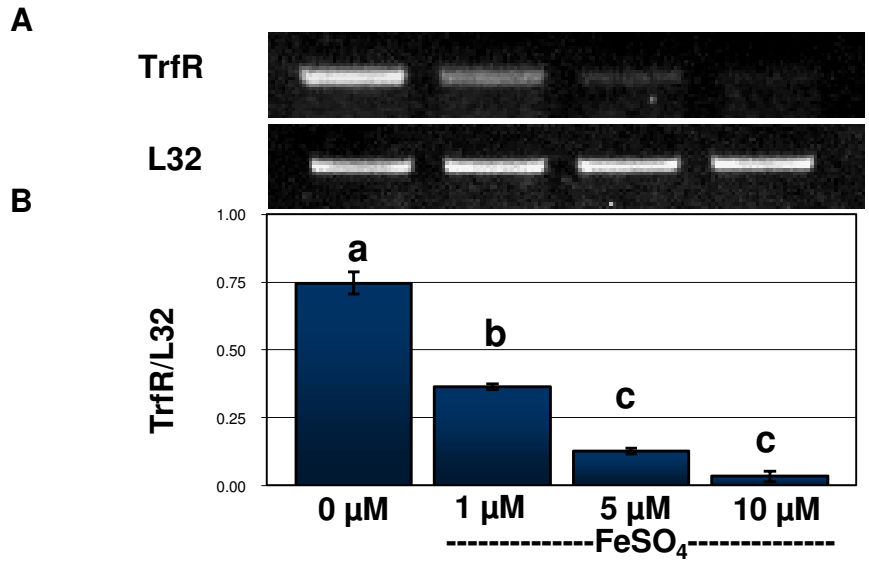

Figure 3: Transferrin receptor (TrfR) gene expression on D21 of cell culture. (A) Representative gel of TrfR and ribosomal L32 gene expression. (B) TrfR gene net intensities were normalized to an internal control, ribosomal L32 mRNA. Net intensity results represent the mean +/- SEM of one sample, each sample ran in triplicate. Results are representative of three independent studies. Mean differences, determined by Tukey HSD ($p < 0.05$), are indicated by letters.

Study 2

Since both 5 µM and 10 µM $FeSO_4$ doses had maximal suppressive effects on TrfR, the dose-reponse relationship of lower $FeSO_4$ concentrations was determined. The effect of 1-5 µM $FeSO_4$ treatment doses on transferrin receptor gene expression at D15 and D20 of cell culture is presented in Figure 4. Results indicate that at both mid-differentiation (D15) and the late differentiation (D20), there was a significant ($p < 0.05$) main effect of treatment, but there was no significant ($p < 0.05$) main effect of time. TrfR gene expression was markedly suppressed only with the 4 µM and 5 µM $FeSO_4$ treatment compared to control.

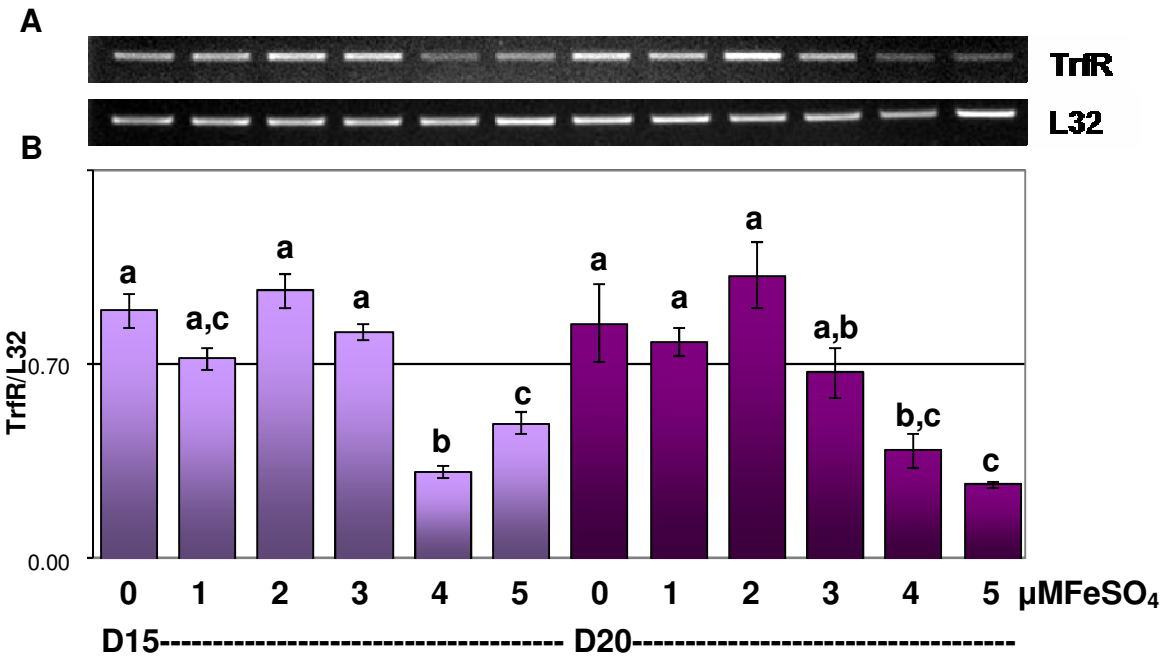

Figure 4: Transferrin receptor (TrfR) gene expression on D15 and D20 of cell culture. (A) Representative gel of TrfR and ribosomal L32 gene expression. (B) TrfR net intensities were normalized to an internal control, ribosomal L32 mRNA. Net intensity results represent the mean +/- SEM of one sample, each sample ran in triplicate. Mean differences, determined by Tukey HSD ($p < 0.05$), within the day are indicated by letters.

Study 1

Protein expression for transferrin receptor and ferritin light-chain was determined (Figure 5). Transferrin receptor protein expression was suppressed with 5 μM $FeSO_4$ and more markedly suppressed with 10 μM $FeSO_4$ treatment in comparison to the 1 μM and control (0 μM) levels (Figure 5A). Ferritin light chain protein expression was higher with the 5 μM and 10 μM $FeSO_4$ treatment doses in comparison to the 1 μM $FeSO_4$ and control (0μM) (Figure 5B).

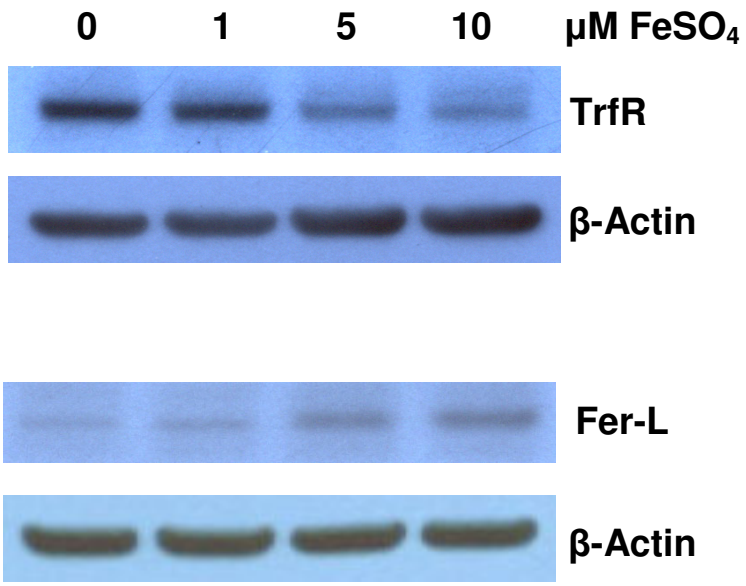

Figure 5: Protein expression of transferrin receptor (TrfR) (A) and ferritin light chain (Fer-L) (B). β-Actin was used as a loading control and determined on the same gel.

Study 2

In order to further examine the time and treatment effects of $FeSO_4$ on transferrin receptor and ferritin light-chain protein expression, the effects of lower $FeSO_4$ doses at two times of culture were then evaluated (Figure 6). Transferrin protein expression was slightly suppressed with 1 μM, 2 μM, and 3 μM $FeSO_4$ and markedly suppressed with 4 μM and 5 μM $FeSO_4$ treatment in comparison to the control (0 μM) at both D15 and D20 of cell culture. There was little ferritin light-chain protein expression evident at D15 of cell culture. Ferritin light-chain protein expression at D20 of cell culture was slightly higher with the 4 μM treatment and 5 μM $FeSO_4$ treatment doses in comparison to all other $FeSO_4$ treatments and control (0μM).

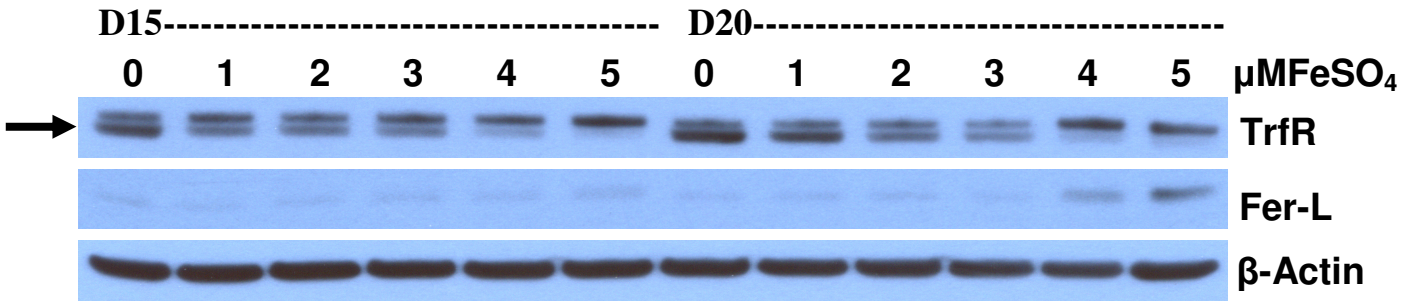

Figure 6: Protein expression of transferrin receptor (TrfR) and ferritin light chain (Fer-L) on D15 and D20 of cell culture. All runs shown are from the same representative gel. TrfR is the lower band, identified with an arrow. β-Actin was used as a loading control. Results are representative of two independent studies.

Effects of Iron Overload on Osteoblast Function

Study 1

Excessive doses of $FeSO_4$ treatment noticeably reduced mineralized nodules and percent mineralized surface area at D21 of cell culture (Figure 7)**.** Alkaline phosphatase-positive colonies (pink) were dramatically reduced with 5 µM and 10 µM $FeSO_4$ and there were no visible mineralized nodules (brown) in wells treated with 10 µM $FeSO_4$ (Figure 7A). Percent mineralized surface area was not significantly different between the 1 µM and control (0 µM) $FeSO_4$ (Figure 7B). There was a significantly ($p < 0.05$) lower percent of mineralized surface area, 75% and 100% lower, respectively, with 5 µM and 10 µM $FeSO_4$ compared to the 1 µM and control (0 µM). There were also significant ($p < 0.05$) differences between the 5 µM and 10 µM $FeSO_4$ dose, the 10 µM $FeSO_4$ dose.

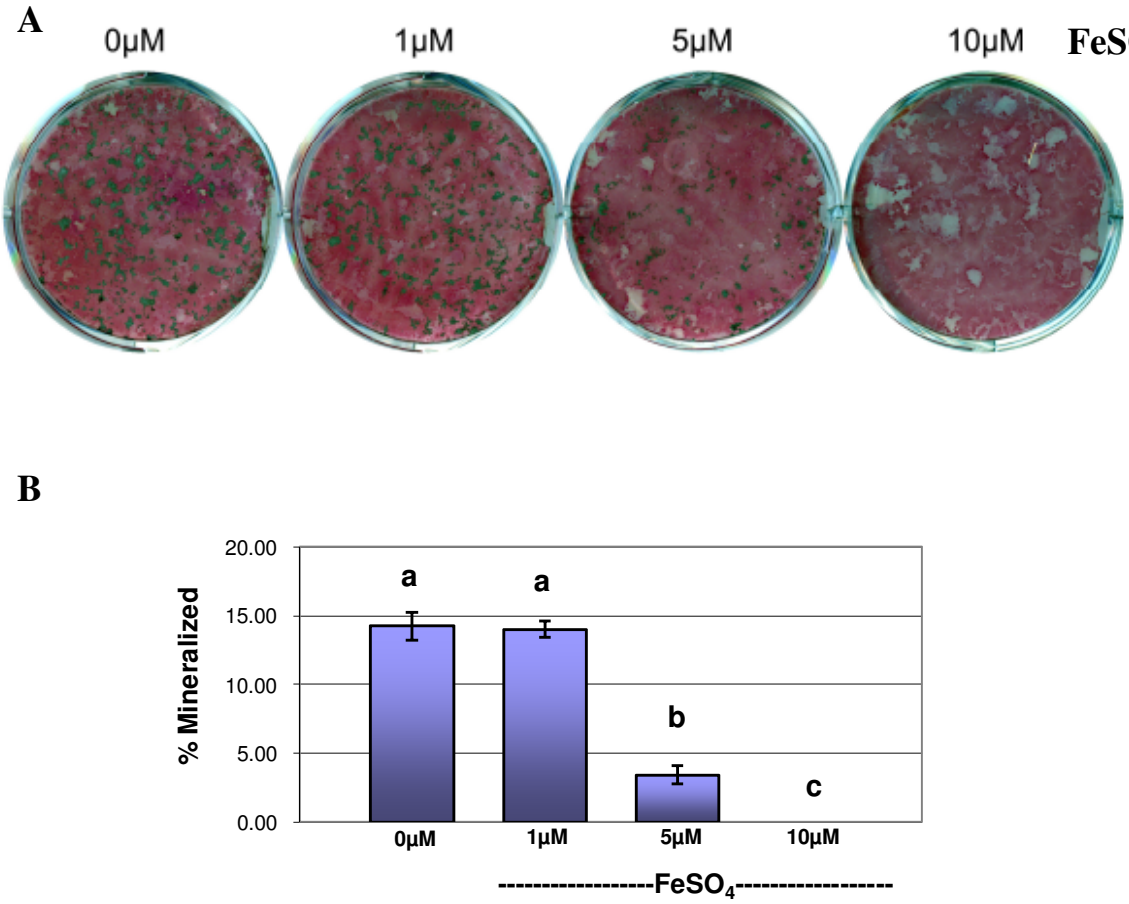

Figure 7: Mineralized nodule formation and percent mineralization of osteoblast-like cells isolated from fetal rat calvaria at D21 of cell culture. (A) Representative stained wells showing alkaline phosphatase positive-colonies (pink) and mineralized nodules (brown)**.** (B) Percent of cell culture dish covered with mineralized nodules. Results represent the mean +/- SEM, n = 3 wells per treatment from one study. Representative results of three independent studies. Mean differences, determined by Tukey HSD ($p < 0.05$), are indicated by letters.

Study 2

In order to further evaluate the dose-response between $FeSO_4$ treatment and mineralization smaller increments in $FeSO_4$ treatment doses were evaluated (Figure 8). Visually there was a slight suppression at D20 in mineralized nodule formation and alkaline phosphatase-positive colonies with both 2 µM and 3 µM treatments compared to 0 µM and 1 µM treatments (Figure 8A). In comparison, even more dramatic reductions were seen with the 4 µM and 5 µM $FeSO_4$ treatment doses and there were no visible mineralized nodules in both compared to all treatment doses and 0 µM (control). There were no significant differences in percent mineralized surface area between the 1 µM $FeSO_4$ dose and 0 µM (control) (Figure 8B). Percent mineralization was about 30% ($p < 0.05$) lower for the 2 µM and 3 µM $FeSO_4$ treatments compared to 0 µM and 1uM treatment doses. Percent mineralized surface area with the 4 µM and 5uM $FeSO_4$ doses were 90 to 100% lower than the control, but there was no significant difference between these treatment doses ($p < 0.05$).

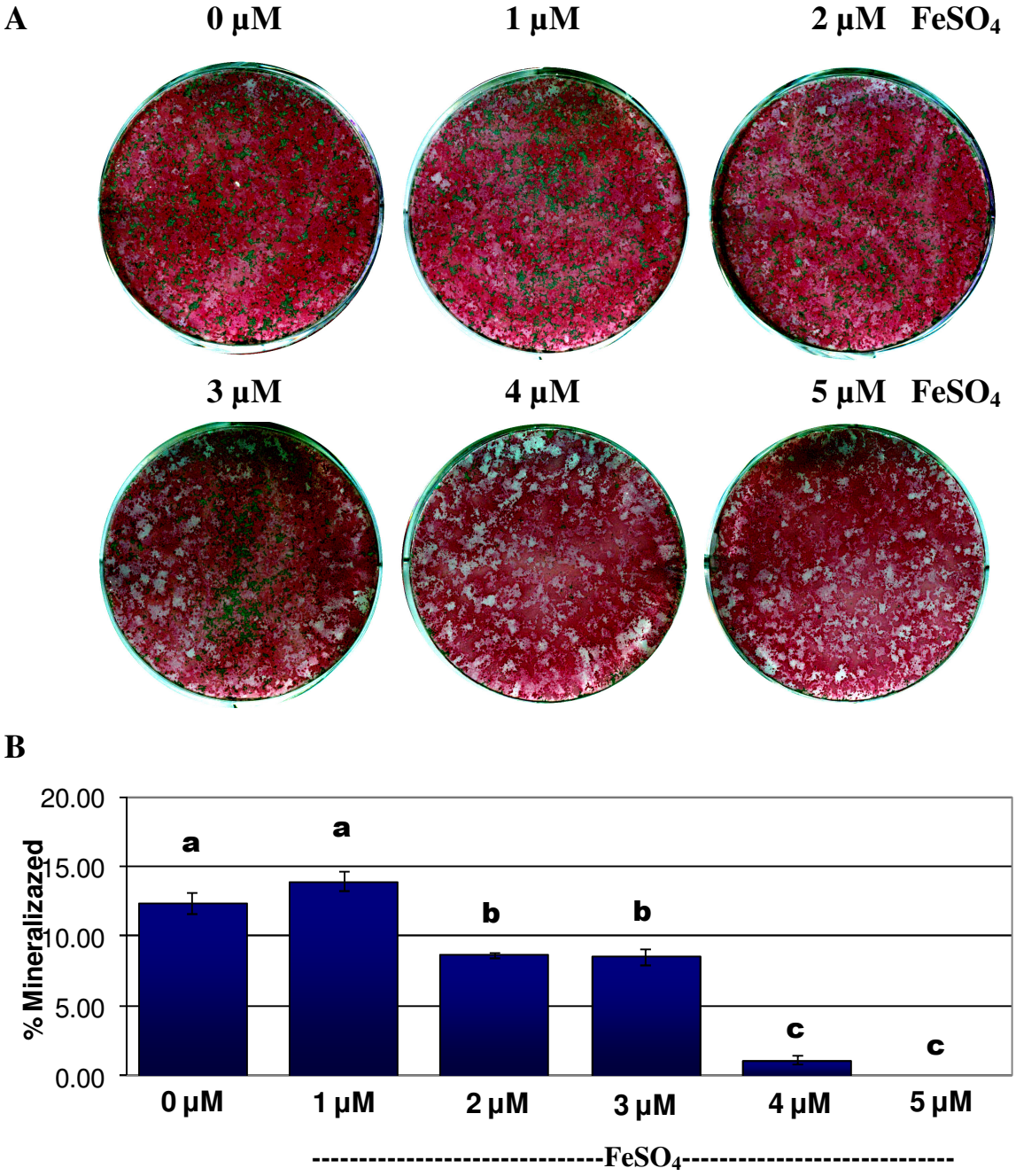

Figure 8: Mineralized nodule formation and percent mineralization of osteoblast-like cells isolated from fetal rat calvaria at D20. (A) Representative stained wells showing alkaline phosphatase positive-colonies (pink) and mineralized nodules (brown)**.** (B) Percent of cell culture dish covered with mineralized nodules. Results represent the mean +/- SEM, n = 4-10 wells per treatment. Mean differences, determined by Tukey HSD ($p < 0.05$), are indicated by letters.

The Effects of Iron Overload on Osteoblast Maturation

Study 1

Excessive $FeSO_4$ treatment suppressed osteoblast phenotypic gene expression of alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteocalcin (OCN) at D21 of cell culture (Figure 9). The 5 μM and 10 μM $FeSO_4$ treatment doses had a pronounced suppressive effect on all osteoblast phenotypic genes in comparison to the 0 μM (control) and 1 μM $FeSO_4$ treatment doses (Figure 9A). The osteoblast phenotypic gene expression of ALP, BSP, and OCN were suppressed with 5 μM and more dramatically suppressed with 10 μM $FeSO_4$ when compared to the 0 μM (control) (Figure 9B). ALP was the only gene suppressed with 1 μM $FeSO_4$ treatment.

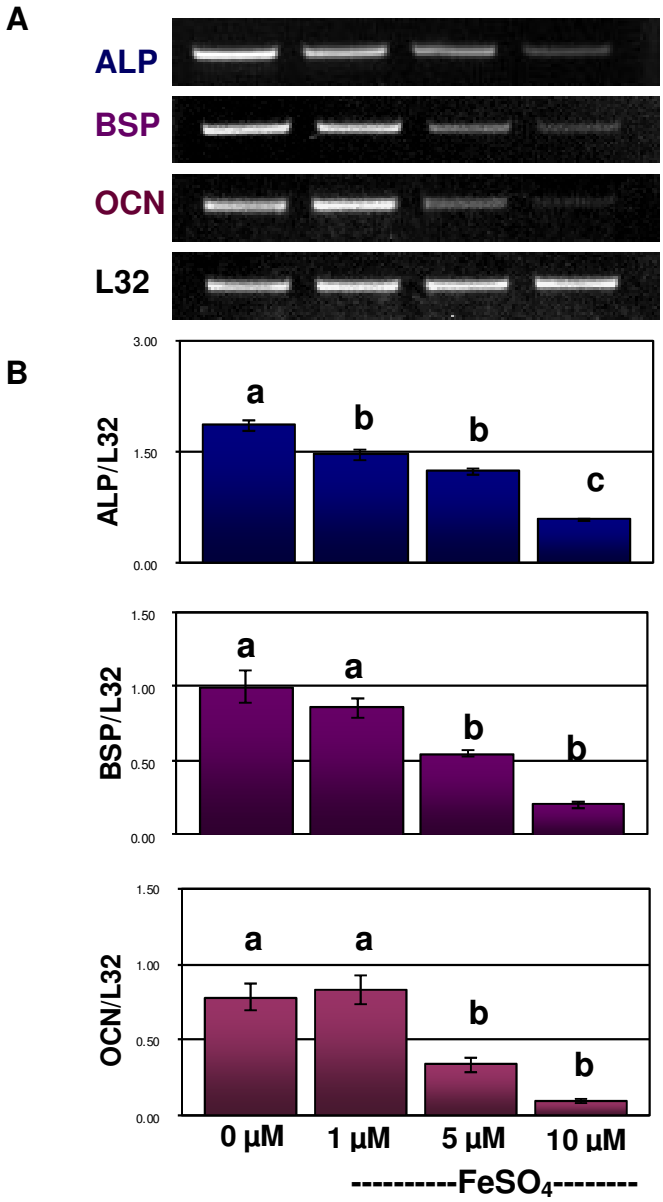

Figure 9: Gene expression of alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteocalcin (OCN) on D21 of cell culture. (A) Representative gel presenting the gene expression of ALP, BSP, OCN, and ribosomal L32. (B) ALP, BSP, and OCN net intensities were normalized to an internal control, ribosomal L32 mRNA. Net intensity results represent the mean +/- SEM of one sample, each run in triplicate. These results are representative of three independent studies. Mean differences, determined by Tukey HSD ($p < 0.05$), are indicated by letters.

Study 2

The incremental effect of $FeSO_4$ on osteoblast phenotype at mid and late differentiation (D15 and D20 of cell culture) was examined. Excessive $FeSO_4$ treatment suppressed osteoblast phenotypic gene expression of alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteocalcin (OCN) on both D15 and D20 of cell culture (Figure 10). ALP gene expression was not markedly altered at any $FeSO_4$ dose at D15 or on D20 in comparison to the control treatment (0 μM). BSP gene expression was markedly suppressed with 5μM $FeSO_4$ treatments doses at D15 and D20 in comparison to the control (0 μM). OCN gene expression was dramatically suppressed with the 4 μM and 5 μM $FeSO_4$ treatment in comparison to all other treatments and control levels (0 μM) at both D15 and D20.

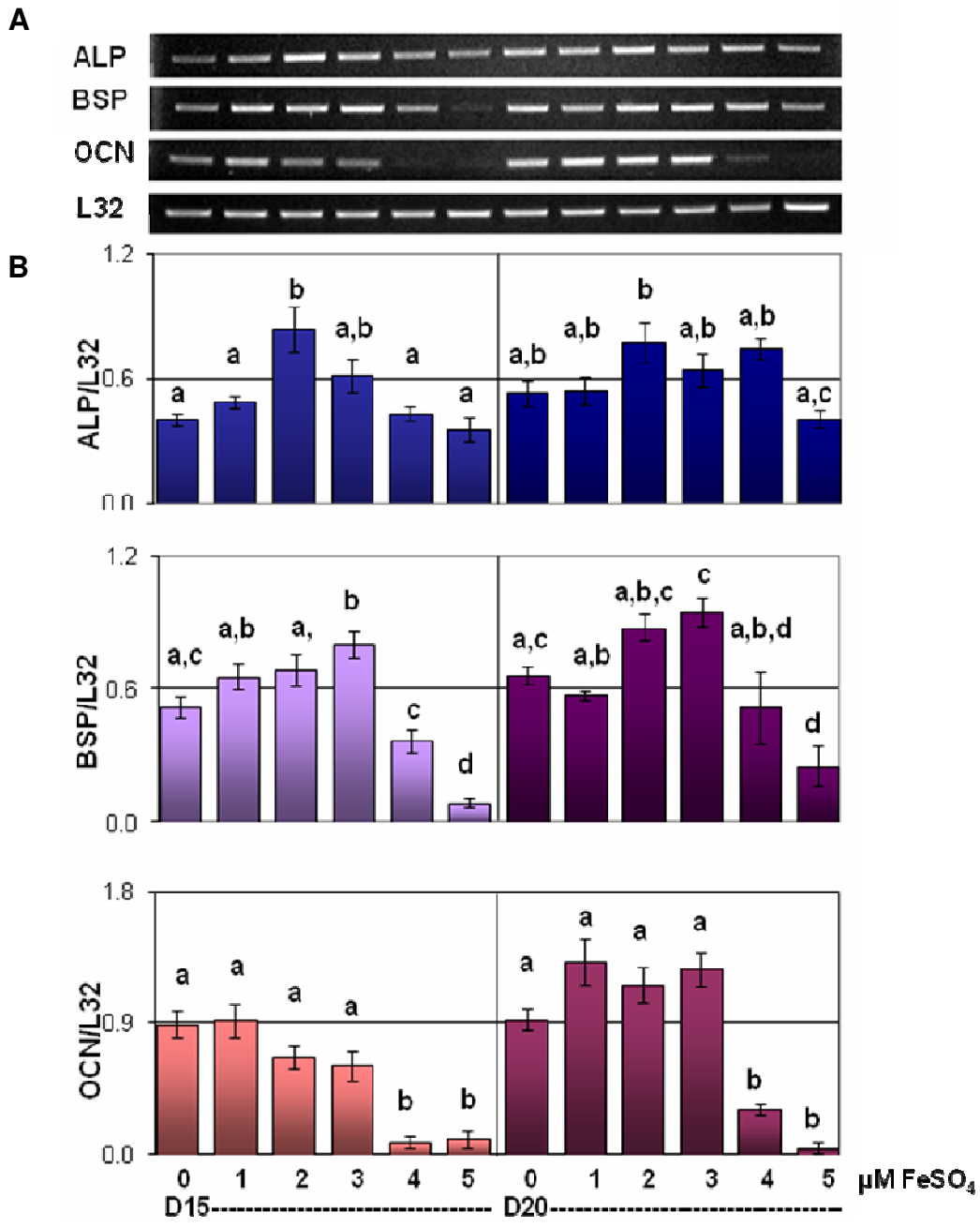

Figure 10: Gene expression of alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteocalcin (OCN) on D15 and D20 of cell culture. (A) Representative gel presenting the gene expression of ALP, BSP, OCN, and ribosomal L32. (B) ALP, BSP, OCN net intensities were normalized to an internal control, ribosomal L32 mRNA. Net intensity results represent the mean +/- SEM of one sample, each run in triplicate. Mean differences, determined by Tukey HSD ($p < 0.05$), within the day are indicated by letters.

Discussion

This is the first report of iron accumulation in an osteoblast cell culture, and the relationship of iron overload to altered iron-regulated gene and protein expression and suppressed osteoblast maturation and function. Intracellular iron concentration levels were shown to increase with excessive $FeSO_4$ treatment, demonstrating iron accumulating ability. The 4 μM $FeSO_4$ treatment dose resulted in near maximal intracellular iron, while maximum intracellular iron levels were reached with 5 μM and 10 μM $FeSO_4$ treatments. These intracellular iron changes occurred at the same $FeSO_4$ doses that markedly altered transferrin receptor and ferritin light chain, and suppressed osteoblast phenotypic maturation and function. Thus results suggest that iron-regulatory proteins in osteoblasts are influenced by intracellular iron concentration during differentiation and that there is an inverse relationship between intracellular iron accumulation and osteoblast maturation and function. These results also suggest that iron excess may be important to consider when assessing bone health.

Although the effect of excessive iron treatment on intracellular iron concentration has not previously been reported in osteoblast-like cells, other cell systems have been evaluated. A study by Hoepken et al. (2004) reported that excessive iron treatment in astrocytes increased intracellular iron in a time- and concentration-dependent manner. Although maximum levels in the study by Hoepken et al. (2004) were not reported, intracellular iron was reported to be 5- and 17-fold higher than the control (0 μM). Non-transferrin-bound iron loading in cultured rat myocardiocytes and human hepatocytes has also been reported to increase intracellular iron concentration levels (Iancu et al. 1987,

Parkes et al. 1993, Cable et al. 1998). Results of these previous studies are consistent with the current study, indicating that intracellular iron concentration increased with increasing $FeSO_4$ treatment. The excessive doses used in the current study were approximately 1.5-fold to 3-fold (4 μM, 5 μM, and 10 μM $FeSO_4$ treatment) higher iron concentration than the control dose (0 μM). Hemochromatotic patients have been reported to have serum iron levels that are 2- to 3-fold higher than normal healthy iron serum values (Jacobs et al. 1972, Bacon 2001). Thus, it is conceivable that intracellular iron concentration in osteoblasts increase *in vivo* with increases in serum iron levels.

Previous studies have reported that osteoblast-like cells isolated from chick embryo tibia and fetal rat calvaria, along with UMR-106-01 osteoblast-like cells, express the iron-regulated proteins transferrin receptor and ferritin, light and heavy chain, during differentiation in vitro (Gentili et al. 1994, Kasai et al. 1990, Spanner et al. 1995). Ferritin light chain is more prevalent in tissues that require long term storage, such as the spleen and liver. Heavy chain ferritin levels are higher in tissues that require a rapid need for iron, such as the heart. In osteoblasts ferritin light chain has been shown to be more prevalent in comparison to ferritin heavy chain (Spanner et al. 1995). Decreased transferrin receptor gene and protein expression and increased ferritin light chain protein expression in response to excessive iron treatment has not previously been reported in osteoblast-like cells. These results are consistent with the results of *in vitro* studies using hepatocytes and astrocytes, where iron overload down-regulated transferrin receptor and up-regulates ferritin light chain gene and protein expression (Cairo et al. 1994, Hoepken et al. 2004, Siah et al. 2006). Human and animal studies on iron overload have reported

the same results; these *in vivo* studies also reported that there were no changes in ferritin heavy chain gene and protein expression (Pietrangelo et al. 1990, Lu et al. 1991, Pietrangelo et al. 1991, Pietrangelo et al. 1992, Cairo et al. 1994).

It is well established that the immature or precursor form of transferrin receptor (86 kDa) go through glycosylation (93 kDa) in the endoplasmic reticulum and homodimers (172 kDa) are further formed by disulfide bridges. The glycan chains further develop into olgliosacharides, creating mature transferrin receptor homodimers (190 kDa), after passing through the Golgi apparatus (Van Driel et al. 1985, Yang et al. 1993). These mature forms can potentially be altered through phosphorylated or fatty acylation (Van Driel et al. 1985, Jing et al. 1990, Beauchamp et al. 1994). This suggests that the lower band or lower molecular weight band in the transferrin receptor Western blot analysis is representative of a less glycosylated form of transferrin receptor. Results presented here would suggest that transferrin receptor is not being posttranslationally modified during excessive iron treatment. The upper band or heterogeneously glycosylated form is increased with excessive iron treatment. This suggests that the mature form of transferrin receptor has reached elevated levels, but that precursors in turn are being down-regulated.

In this study, osteoblast phenotypic development and function are dramatically reduced in response to increasing $FeSO_4$ treatment. Although previous studies on the effects of excessive iron on osteoblast function and maturation *in vitro* have not been reported, these effects *in vivo* have been reported. Animal and human studies have reported links between iron overload and decreased BMD, lower serum osteocalcin,

delays in bone mineralization, lower bone formation, and lower osteoblast number (Vernejoul et al. 1984, Matsushima et al. 2001, Domrongkitchaiporn et al. 2003, Mahachoklertwattana et al. 2003, Guggenbuhl et al. 2005, Anelopoulos 2006, Salama et al. 2006).

Further studies are needed to determine potential mechanisms of iron overload effects on osteoblast maturation and function. Oxidative stress and the induction of free radicals from labile iron, also known as "redox-active" or "chelatable", is a likely contributor. Excessive intracellular labile iron has been reported to heighten oxidative stress in rat hepatocytes and Jurket cells (Barbouti et al. 2001, Rauen et al. 2004). A novel finding in the current study was the evaluation of increasing intracellular iron concentration levels with excessive $FeSO_4$ treatment. However, the proportion of intracellular iron that was free versus bound iron was not determined. Intracellular iron concentration contributes to all bound or unbound iron within the cell and further analysis of only redox-active iron would provide a clearer insight into potential mechanisms.

Human studies have reported that oxidative stress negatively affects BMD (Maggio et al. 2003, Hall et al. 1998, Leveille et al.1997, Basu et al. 2001). Evidence *in vitro* also suggests that ROS alter osteoblast function. Arai et al. (2007) using MC3T3-E1 cells treated with H_2O_2, evaluated the effects of oxidative stress on osteoblast mineralization. Nodule formation and the gene expression of alkaline phosphatase, bone sialoprotein, and runt-related transcription factor 2 (Runx2), a key activator of osteocalcin transcription, were suppressed in H_2O_2-treated cells when compared to non-treated cells. A further study by Hinoi et al. (2006) reported that Nrf2 (nuclear factor E2

p45-related factor 2) alters Runx2 transcription and lowers osteoblast differentiation. Significantly lower levels were reported in alkaline phosphatase activity and the osteoblast differentiation gene markers Runx2, bone sialoprotein, and osteocalcin in Nrf2 transfected MC3T3-E1 cells in comparison to non-transfected cells. The overexpression of Nrf2 was reported to significantly lower Runx2-dependent stimulation of osteocalcin gene 2, a mouse osteocalcin promoter. This study, in addition with Arai et al. (2007), supports the role of Nrf2 on altering Runx2, ultimately resulting in lower osteoblast differentiation.

Oxidative stress has been shown to play a major role in suppressing osteoblast differentiation by triggering extracellular signaling pathways. Bai et al. (2004), using primary rabbit calvarial osteoblast and bone marrow stromal cells treated with H_2O_2, reported that oxidative stress stimulated the extracellular signal-regulated kinases (ERK) and nuclear factor-κB (NFκB) signaling pathways. Treated cells had dramatically lower alkaline phosphatase staining, alkaline phosphatase protein expression, and osteoblast colony forming units in comparison to non-treated cells. Treated cells also had dramatic suppression of type I collagen and inhibition of nuclear Runx2. Bone morphogenetic protein-2, an initiator of Runx2 transcription, and Runx2 protein levels were not suppressed in treated cells compared to controls. This study experimentally blocked NFκB and ERK pathways and reported a decreased suppression of nuclear Runx2 and type I collagen protein expression, thus confirming the involvement of these pathways in inhibiting osteoblast differentiation in response to oxidative stress.

In conclusion, this study presents novel findings that excessive $FeSO_4$ treatment increased intracellular iron, altered key iron-regulated gene and protein expression, and suppressed osteoblast maturation and function in osteoblast-like cells isolated from fetal rat calvaria in a dose-dependent manner. Findings of this study provide a basic foundation for further examining potential mechanisms for which iron overload affects the skeleton and suggests that iron excess may be important to consider when assessing bone health.

CHAPTER IV

EPILOGUE

The gene and protein expression of transferrin receptor and ferritin light chain were altered with excessive iron treatment. Although these are novel findings in osteoblast-like cells isolated from fetal rat calvaria, further analysis of other iron-regulated proteins will provide more insight into potential mechanisms. For example, divalent metal transporter is another iron-regulated protein that has been reported to be involved in iron uptake by enterocytes and astrocytes (Morgan et al. 2002, Erikson et al. 2006). This transporter has not been previously evaluated in osteoblast-like cells and would be a potentially novel addition to the current study. Alterations in iron-regulated gene and protein expression with acute excessive iron treatment are another aspect that would provide more insight into time-dependent effects. The acute excessive iron treatment effects on key iron-regulated gene and protein expression of transferrin receptor, ferritin light chain, and ferritin heavy chain with osteoblast-like cells isolated from fetal rat calvaria are currently being evaluated in our lab.

Links between oxidative stress and the suppression of osteoblast development have been reported by Bai et al. (2004) and Arai et al. (2007). These studies reported that increased oxidative stress in osteoblasts induced by H_2O_2 suppressed the key osteogenic

marker gene expression of alkaline phosphatase, bone sialoprotein, type I collagen, and Runx2. Although animal study findings have shown a relationship between iron overload, oxidative stress, and decreased BMD, further analyses of oxidative stress in relation to iron overload and osteoblast development *in vitro* is still needed. Further *in vitro* analysis might be conducted by evaluating the effects of iron overload on oxidative stress markers. Glutathione peroxidase is one example of an antioxidant that is released from osteoblasts (Isomura et al. 2004). Alkaline phosphatase and bone sialoprotein gene expression were also reported in the current study to be suppressed with excessive iron treatment, but type I collagen and Runx2 were not evaluated. An analysis of these genes in relation to excessive iron and osteoblasts would be a potential next step in evaluating alterations in other osteogenic markers.

Cellular death by apoptosis or necrosis may also be induced in response to iron overload. An evaluation of acute apoptotic effects of iron overload on osteoblast-like cells isolated from fetal rat calvaria is currently underway. Cell death via apoptosis can be initiated by a multitude of processes, but two specific pathways are through the intrinsic pathway or mitochondria's release of cytochrome-c and the extrinsic pathway or by "death" receptors (Ueda et al. 2002). Studies *in vitro* have reported that iron overload induces apoptosis predominately by the intrinsic pathway (Doulias et al. 2003, Rauen et al. 2004). Cytochrome-c release from the mitochondria is one initiator of caspases, specifically caspase-3 and caspase-9 (Ueda et al. 2002). These active and inactive forms of these proteins are currently being evaluated in our lab, along with other early indicators of apoptosis such as DNA fragmentation and phosphatidyl serine relocation.

The extrinsic pathway and necrosis are other potential directions that could be further analyzed. The extrinsic pathway is initiated through the Fas death domain protein and in turn activates caspases 8 and 10. Caspase-8 can initiate the release of cytochrome-c by activating Bid which is part of the pro-apoptotic Bcl-2 family (Ghobrial et al. 2005). Reactive oxygen species have been reported to inhibit caspase-9 and cause necrosis instead of apoptosis (Ueda et al. 2002). An assessment of elevated cellular death could further support potential mechanisms correlating iron overload to the suppression of osteoblast development.

There are inherent limitations to *in vitro* studies, including cell culture conditions. First, extending the length of cell culture past day 21 may have provided insight into the nature of the effects of iron. The suppression of osteoblast function and maturation might be "rescued" after this time frame if the iron-related effects were due to a delay rather than a suppression of osteoblast maturation. The detachment of cells from the plate and overgrowth of cell layers at the end of culture (~D20-21) made this analysis difficult. Second, iron overload may also interfere with mineralization per se of the multilayered nodules, used as an *in vitro* indicator of osteoblast function. In iron overloaded cells, multilayering was evident but little was mineralized. Thus, a separate quantification of unmineralized nodules would be informative. Previous studies have reported that iron overload in humans does not affect serum calcium levels (Wardle et al. 1969, Salama et al. 2006). However, animal studies have reported that urinary calcium levels increase with dietary iron overload (Matsushima et al. 2001). Due to the major role of calcium in bone mineralization, analysis of calcium levels may also be informative.

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APPENDIX. Iron Concentration.

Table 2 : Media and reagent iron concentration determined by Graphite Furnace Atomic Absorption Spectrometry.

Media Plus Treatment Doses*	**µmol/L**	**Components of Media Solution**	**µmol/L**
0 µM (Control)	0.0031	α – Modified Eagles Medium	0.0003
1 µM	0.0041	Fetal Bovine Serum[1]	0.0315
2 µM	0.0049	Dexamethasone	0.0006
3 µM	0.0057	Phosphate Buffer Saline	0.0002
4 µM	0.0067	Nitric Acid	0.0002
5 µM	0.0072	Deionized Water	0.0000
		β – Glycerol Phosphate	0.0017
		Antibiotics	0.0003

[1] Manufacturer value: 0.0291 µmol/L, * Media components listed in right column