COVER SHEET

TITLE: The effects of erythropoietin on transferrin receptor concentration in rat duodenum tissue

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Effects of iron deficiency and enteral erythropoietin on transferrin receptor concentration in newborn rat duodenum

Preliminary studies showed factors contained in human milk retain their mitogenic activity following pasteurization. Iron is critical in cell proliferation in early life. Erythropoietin (Epo), a factor found in human milk, stimulates iron utilization, but its role in iron absorption is unclear. We hypothesized that expression of duodenal iron transporter, TfR, would be higher in damfed (DF) or rats with iron deficiency anemia (IDA) fed enteral Epo from postnatal day 4-12, compared to control. Duodenum and liver were stained for iron and duodenal TfR immunohistochemistry was performed. Body or liver iron content was measured. TfR density was higher in IDA, compared to DF ($p=0.05$). Liver iron content was greater in IDA and IDA+Epo than Dam or Dam+Epo, $p<0.0005$, but liver Prussian blue staining was lower in IDA and IDA+Epo, compared to Dam or Dam+Epo ($p<0.0001$). These data support increased TfR density with iron deficiency, but no appreciable effect with Epo.

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Experiment I: Pasteurization does not alter the mitogenic activity of biologically active factors in human milk on fetal gastrointestinal cells

Introduction

The many benefits associated with feeding human milk to infants are widely documented [1,2,3]. Studies have consistently found reduced risk of gastroenteritis, respiratory infections, and immunological diseases and increased cognitive development among breast fed infants compared to their formula fed peers [4]. Initial concern regarding the transmittance of harmful pathogens from mother to infant during feeding has led to the increase in pasteurization and development of pasteurized milk banks [5]. When approaching the nutritional needs of premature infants who display underdeveloped immune defense systems, pasteurization is even more attractive [2]. Pasteurization of both donor milk and mother's own milk for very low birth weight infants has become increasingly popular in NICUs [5]. Several studies have verified the ability of standard Holder pasteurization techniques in effectively eliminating most viruses and bacteria contained in milk [7,8,9].

In addition to traditionally recognized macro and micronutrients, human milk is also a significant source of many biologically-active factors, which are not available in formula [10,4]. These proteins including erythropoietin, transferrin, epidermal and insulin-like growth factors, and prolactin are important for infant intestinal growth and development [1,11]. The neonate's developing intestinal tract has low proteolytic activity, which allows for greater absorbance of these factors [10]. Once absorbed, biologically-active factors may be available to function in the infant [4].

Studies have illustrated the growth promoting effects of milk factors on fetal intestinal cells where a positive correlation between growth and milk dose was observed [12]. These effects illustrate the importance of biologically-active factors contained in milk, which exert their effects on the intestine, are absorbed by receptors on luminal epithelial cells, and diffuse into the blood stream [11,13]. The development initiated by such factors is important as the infant's gastrointestinal tract adapts to extrauterine nutritional requirements [12].

In clinical practice today, premature infants receive their mother's expressed milk or milk from unrelated donors from milk banks. Pasteurization has become a mandatory process for donated milk in banks; however, it remains an optional procedure for mothers feeding their own children. In order to determine the advantages of pasteurizing all milk, it is important to determine what nutrients in milk are altered by pasteurization [14].

Pasteurization does not appear to affect the amount of macronutrients in human milk [15]. Initial concern for the viability of biologically-active factors following the high temperatures of pasteurization has been lessened after several studies showed no major structural degradation of biological factors. Research in bovine milk showed that insulin-like growth factor-I was measured as intact following pasteurization [16]. Similarly, several studies on pasteurized human milk revealed little to no significant changes in concentrations of growth factors and enzyme components of milk samples [2], and protein concentrations were found to be unaffected by pasteurization [17]. However, both bile salt-stimulated lipase and lipoprotein lipase were determined to be completely inactivated following Holder pasteurization [5].

Because factor concentrations are generally measured with immunoassays that are designed to measure a fraction of the physical structure of proteins, it is unknown whether heat pasteurization may cause subtle structural changes resulting in impaired function of the protein's receptor site. We hypothesized that human milk samples would retain their mitogenic effects

following pasteurization without limited break down of tertiary protein structure. This hypothesis was tested by measuring cell growth of an *in vitro* fetal gastrointestinal cell model.

Fetal intestinal cells were chosen as a suitable model for this research because milk-borne bioactive proteins exert their biologic effects on the intestine and are absorbed into the blood stream by passage through these cells [11]. In addition, previous studies have shown that fetal gastrointestinal cells grow in the presence of human milk [12].

Methods

Sample Collection and Pasteurization

Human breast milk samples were collected at Meriter Hospital in Madison, Wisconsin after approval from the Human Subjects Committee. Each sample was stored at -80^{o}C. After thawing, samples were divided into pasteurized and unpasteurized portions. Pasteurization was achieved by heating samples to 62.5°C for 30 minutes. All samples were stored at -80°C until testing. Samples used in this study are also being used by Andrea Willeitner, MD, who is conducting large scale studies of how heat affects the bioactive factors in human milk.

Cell Culture

Fetal FHs 74 small intestine cells (ATCC, Manassas, VA) were chosen as the cell model for analysis. Cells were plated in 96-well flat bottom tissue culture plates (Linbro, Solon, OH) and were grown in Hybri-Care Medium 66-X (ATCC, Manassas, VA), prepared to a pH of 7.4. One percent Penicillin-Streptomycin-Glutamine 100X (Gibco, Carlsbad, CA), 0.3% Recombinant Human Epidermal Growth Factor 236-EG (R&D Systems, McKinley Place, NE), and 10% Fetal Bovine Serum (FBS) 30-2020 (ATTC, Manassa, VA) were added to the growth medium.

Once cells reached a concentration of 1.25E5 cells per mL, or about 80% confluency, the growth medium was removed, wells were washed with sterile PBS (Sigma-Aldrich, St. Louis, MO) and a two percent concentration of human milk prepared in growth medium without FBS was added. This milk concentration was based on a desired intermediate fetal gastrointestinal cell growth rate which would allow for identification of any inhibitory or enhancing effects of milk addition [12]. Cells were incubated at 37°C for 44 hours in the milk. Plates contained paired pasteurized and unpasteurized samples from each milk donor to enable accurate comparison of the effects of heat pasteurization.

Following the incubation period, a Thiazolyl Blue Tetrazolium Bromide (MTT) Analysis was used to measure cell mitochondrial activity and cell proliferation. Cells were incubated in 50 μL of 2g/L MTT solution for two hours, medium was removed and 50μL of DMSO was added to each well. Spectrometry was utilized to assess absorbance at 570nm [18]. During the MTT assay growth media was used as a negative control, and complete growth media with 15 percent fetal bovine serum (FBS) served as a positive control. Media with 15 percent FBS should produce maximal growth conditions for cells *in vitro*.

Statistical Analysis

Spectrometry readings at 570 nm were corrected by subtracting the absorbance of blank wells containing only DMSO. Results of pasteurized and unpasteurized samples were statistically analyzed using a paired t-test. P values of 0.05 or lower were considered statistically significant, supporting that there was a less than 5% probability that findings were due to chance.

Results

The results of 18 paired samples were summarized. A P-Value of .6417 revealed no statistical difference in cell proliferation after treatment with pasteurized and unpasteurized milk (Figure 1). However, mean values for both pasteurized (0.187) and unpasteurized samples

(0.1778) were significantly higher than the negative control (0.127) and significantly lower than that of the positive control (0.271).

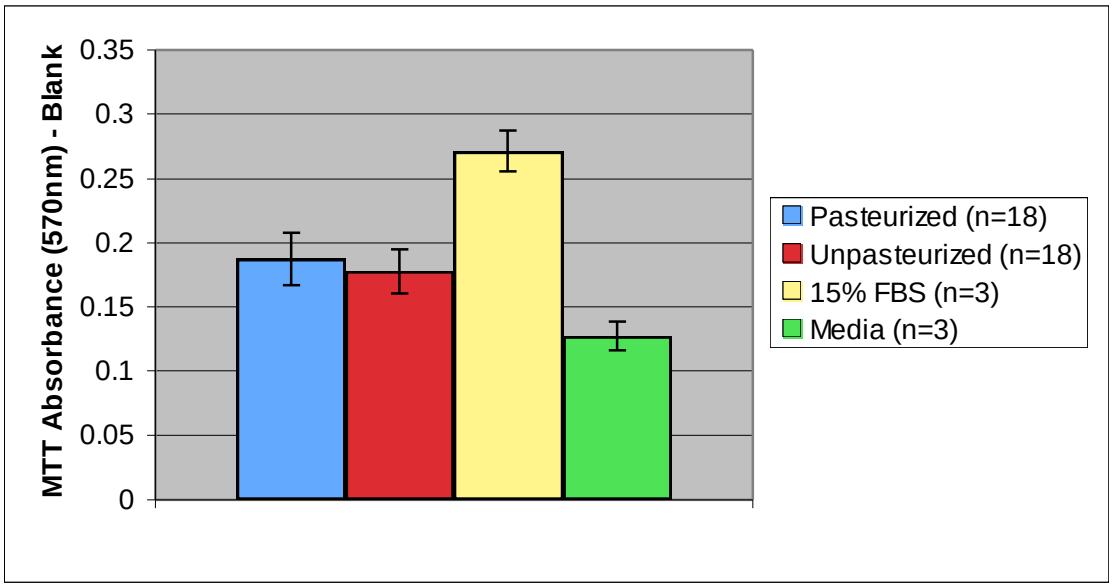

Figure 1: Corrected Thiazolyl Blue Tetrazolium Bromide analysis results. Fetal FHs 74 small intestine cells were incubated in 2% pasteurized or unpasteurized milk for 48 hours prior to the assay. Absorbance was read at 570nm and corrected with the absorbance of a DMSO blank.

Conclusions

Based on our hypothesis, we anticipated minimal difference in growth between cells treated with pasteurized and unpasteurized milk samples. Results of MTT analysis supported this hypothesis, as no statistical difference in cell growth among pasteurized and unpasteurized samples was observed. Thus, these data illustrate that pasteurization does not appear to decrease the mitogenic activity of milk in an *in vitro* bioassay. More sensitive tests of these results would be beneficial. A Bicinchoninic Acid Protein Assay would allow us to assess if protein levels are effected by pasteurization. Further, utilizing a hemocytometer to count cells after milk incubation would provide a direct measure of cell proliferation.

These results suggest that the presence of protective factors such as chaperone proteins and the high carbohydrate content of biologically-active factors, known to be present may enable them to resist to heat degradation. This is plausible, as these same mechanisms allow biologically-active proteins to resist acid degradation *in vivo* [19]. Such properties are illustrated in studies that have shown I-EGF's ability to resist gastric acid degradation *in vitro* [10].

However, the ability of milk constituents to retain their function following pasteurization seems to vary among factors. Decreased activity following pasteurization has been reported for several biologically active factors [20]. Yet, the data reported here supports that the mitogenic effects of human milk are retained after pasteurization. It is possible that although concentrations of factors are reduced by pasteurization, they remain in adequate amounts to exert their biological function [21].

The data collected in this experiment has important implications for infant nutrition. Pasteurization is known to effectively eliminate pathogens transferred in milk [9,8], and these results suggest that mitogenic growth factors are not inhibited when subjected to the temperatures of pasteurization. Therefore, pasteurized milk from milk banks may provide

infants with an optimal nutritional food source containing carbohydrates, proteins, and growth factors, without the risk of pathogen transfer. This is especially important for premature infants who possess underdeveloped immune systems and are more susceptible to illness from unpasteurized milk. However, it will be important to assess the ability of specific biologically-active factors following pasteurization, to ensure that other beneficial factors are still transferred to the infant.

The results of our pasteurization study suggest that biologically-active factors with mitogenic effects are accessible through human milk. We became interested in how factors in milk are associated with duodenal iron absorption. Iron is transferred from mother to fetus during the last trimester of pregnancy [22], and iron deficiency is common in premature infants. After birth, iron's presence and transport is correlated with two biologically active factors found in milk, transferrin and erythropoietin. Transferrin, a serum protein with two iron binding sites, acts as an iron source for immature red blood cells [23]. Erythropoietin is known to exert trophic effects in the intestinal tract, and promote erythropoiesis. In Experiment II, we sought to explore the role of erythropoietin in duodenal iron absorption.

Experiment II: The effects of erythropoietin on transferrin receptor concentration in rat duodenum tissue

Introduction

The availability and abundance of iron is an extremely critical factor in the DNA synthesis and cell proliferation necessary for infant growth [24]. The necessity of this need is seen in the immediate arrest of growth and subsequent death in cells where iron is withheld [25]. However, excess iron poses an equally menacing threat due to free iron's ability to create free radicals [25]. Thus, vigilant regulation is necessary in regulating the balance of iron.

Iron Transport

Dietary iron is absorbed at the luminal surface of duodenal epithelial cells [25] which are sensitive to the body's iron needs [22]. Most ingested iron exists in a Fe^{3+} state, and must first be reduced to Fe^{2+} before absorption is possible [25]. This reduction and the formation of iron chelators [22] is facilitated by the low pH of the duodenum that results from the influx of gastric acid from the stomach [27]. Bile, containing substances that are able to chelate and reduce iron, is also secreted into the duodenum [22]. Additionally, duodenal epithelium contain enzymatic ferric reductase to facilitate conversion to the ferrous state [27]. Collectively, these characteristics make the duodenum a suitable site for iron absorption, and studies have verified it as the primary absorption site for iron [34].

Once reduced, ferrous iron enters the enterocyte via a DMT-1 receptor located on the cell's apical membrane [26] (Figure 2). Iron is quickly bound by transferrin (Tf), a protein with high affinity for iron (Hentze et al 2004). Tf is characteristic in multicellular organisms as a means of chelating iron for safe transport between cells [27]. Most extracellular iron circulating in the plasma is also bound to Tf [25]. Tf-bound iron can leave the enterocyte and enter circulation through the ferroportin receptor on the enterocyte's basolateral surface or via transferrin receptor (TfR), which is also located on the basolateral surface of the cell. TfR is able to bind transferrin and endocytosis it into the cell. Once inside, the vesicle fuses with a lysozyme, whose acidic pH promotes iron's release from transferrin, and TfR is recycled back to the cell's surface [28] (Figure 3).

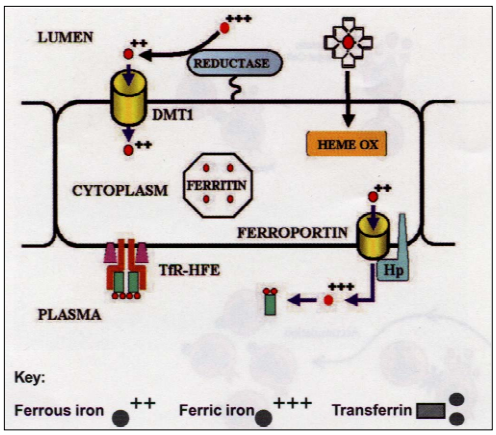

Figure 2: Iron transporters in intestinal epithelial cells. TfR and ferroportin are located on the basolateral membrane, while DMT-1 is on the luminal surface of the cell [29].

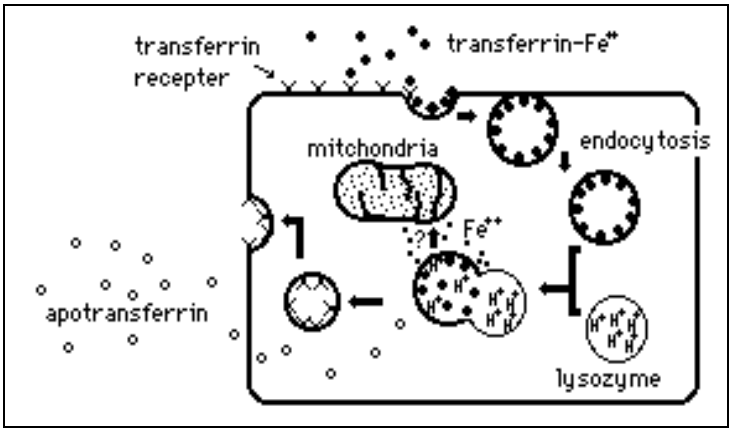

Figure 3: Uptake of Tf by TfR. Transferrin binds transferrin receptor at the top of the diagram. The Tf:TfR complex is endocytosed into the cell where it fuses with a lysozyme. The acidic pH of the lysozyme promotes irons release from transferrin. Free iron may travel to the mitochondria to form heme. TfR is recycled to the cell surface [30].

Regulation of iron absorption and delivery

Delivery of iron from duodenal intestinal epithelial cells to the enteral-hepatic circulation is accomplished in part through TfR. The liver is main site of iron storage within the body [31,27], and is believed to negatively regulate duodenal iron absorption [26]. Hepatocytes control the expression of hemochromatosis (HFE) protein, hemojuvelin (HJV), transferrin receptor 2 (TfR2), and hepcidin [26]. Recently, hepcidin has been cited as a possible iron regulator. The expression and secretion of hepcidin is regulated by HFE, JHV, and TfR2 [26]. Hepcidin is believed to act on enterocytes, hepatocytes and macrophages, by degrading ferroportin to decrease iron export capabilities [26,32].

In addition, several other mechanisms have been suggested as players in the regulation iron levels. In 2000, Andrews proposed that proper iron levels are maintained through the concurrent regulation of dietary intake, a stores regulator that senses saturation of TfR, and an erythropoietic regulator that adjusts intestinal iron absorption with changing erythropoietic needs [27]. Conrad et al maintain that the most important regulator is the rate of erythropoiesis. They observed enhanced iron absorption with increased erythropoiesis [22]. However, other studies have concluded that increased erythropoiesis alone does not affect iron absorption [33].

Iron deficiency increases duodenal iron bind capacities. Conrad et al. reported increased iron binding of intestinal mucosal homogenates in iron-deficient rats compared to control [34]. In addition, an increase in intestinal surface area has been observed with iron deficiency, perhaps in an attempt to increase the area in which iron can be absorbed [22,24].

The concentration of TfR on a cell has also been related to iron delivery. Iron responsive elements (IREs) and iron regulatory proteins (IRPs) monitor intracellular iron levels [25]. When iron becomes scarce, these proteins signal for increased translation of TfR, which allows the cell to increase intracellular iron levels [24]. Thus, the presence of TfR provides a measure of cellular iron as TfR levels are high with deficiency and low with sufficiency [35]. Additionally, Goodnough et al observed increased TfR with both iron deficiency and increased erythropoiesis [36].

Erythropoietin

Iron deficiency is commonly observed in the premature infant [37], and erythropoietin (Epo) has been suggested as a potential agent to increase iron absorption. Epo is a biologically active factor found in human milk. It is known to stimulate erythropoiesis, but its role in iron absorption is unclear [38]. Anemic infants often have low levels of Epo [37].

In utero, the fetus swallows Epo contained in amniotic fluid, and breast fed infant receive Epo in their mother's milk [39,40]. Functional Epo receptors (Epo-R) have been located in the intestinal villi of both humans and rats [39,41]. Concern over Epo's ability to resist degradation in the harsh environment created during digestion have been decreased by several studies that show Epo's retained function in the gut and transfer through the intestinal tract [37,40,42].

Experimentation has revealed a significant decrease in ferritin levels following treatment with Epo, supporting Epo's ability to stimulate iron utilization [43]. One study of human adults with renal failure found a threefold increase in nonheme iron absorption in patients treated with enteral Epo [44]. Kling et al reported increased erythropoiesis when Epo treatment was supplemented with the addition of iron [42]. However, Juul et al. reported no difference in serum Epo concentrations among iron deficient and control rat pups, opposing the use of enteral Epo as a means to increase iron absorption and erythropoiesis [39]. The inconsistency among these results warrants further studies.

Epo does seem to exert trophic effects on the small intestine. Recombinant human Epo promoted proliferation in rat gastric mucosal cells [45]. Further enteral Epo treatment has been correlated with a dose dependent increase in villus length and surface area [39,42]. Currently, Epo is being used as an effective treatment for anemia in adult human patients on hemodialysis and chemotherapy [46,47].

Hypothesis

We sought to explore the potential of enteral Epo as a treatment for iron deficiency in the premature infant. Our aim was to assess the effect of enteral Epo as a means to increase duodenal iron absorption in iron-deficient anemic (IDA) newborn rats. We hypothesized that the expression of duodenal TfR would be higher in dam fed or IDA rats receiving enteral Epo treatments, compared to control. Immunohistochemistry was used to provide a quantitative measurement of TfR concentration in duodenal tissues of newborn rats. Liver iron was examined through H&E and Prussian blue staining.

Methods

Animals

CD IGS Sprague-Dawley rats were purchased after receiving approval from the Animal Care and Use Committee at the University of Wisconsin, Madison. Rat litters were reduced to 10 pups on day of life one.

Feeding and Treatment

Rats were treated from postnatal day 4-12 (P4-P12). Rats were either fed by their mother (Dam) or by administering iron-deficient artificial rat milk substitute formula by gastrostomy (IDA). Artificial formula was prepared and administered analogous to the methods of Dvorak et al. and Kling et al. [48,42]. Recombinant human Epo (Amgen, Thousand Oaks, CA) was administered enterally at a dose of 425U/kg/d. On day of life 12, pups were killed with lethal anesthesia of inhaled isoflurane.

Blood and Tissue Harvest

After sacrifice total body, liver, and duodenal weights were recorded. The duodenum was harvested, cut open, and fixed in formalin, before being embedded in paraffin block. Liver

tissue was collected preserved in formalin, sectioned, placed on microscope slides and treated with Prussian blue iron stain (Sigma-Aldrich, St. Louis, MO) or hematoxylin and EosinY (H&E) (Sigma-Aldrich, St. Louis, MO).

Whole blood was collected via cardiac puncture. White blood cell counts were found with an MD16 counter (Coulter Diagnostic, Hialeah, FL).

Animals designated for body or kidney iron analysis did not have blood draws performed. Body iron content was determined after removing intestinal tracts to eliminate any unabsorbed iron from oral iron supplements remaining in the digestive tract. Carcasses or kidneys were weighed and iron content was determined by acid digestion and atomic absorption. Tissue samples were placed in Teflon® tubes engineered at UW Dept. of Engineering for acid digestion. Nitric acid and then perchloric acid were added for complete digestion. Tubes were brought up to the same volume with 3M HCl [54]. Iron standards, samples, and controls were read using an atomic absorption flame spectrophotometer (Perkin Elmer 2280 Atomic Absorption Spectrophotometer, Waltham, MA, USA).

Duodenal Immunohistochemistry for TfR

After preliminary results yielded a great loss of tissue morphology when frozen tissue was used for immunohistochemistry, paraffin blocks were used for analysis (Figure 4). Twenty-four hours prior to immunohistochemical staining, a microtome was used to cut 3.5 µm sections from paraffin blocks containing duodenal tissue. Two tissue sections from each block were placed on a microscope slide and dried overnight at 37ºC. Tissues were deparaffinized and rehydrated in CitroSolv and several dilutions of ethanol. Antigen retrieval via a six minute microwave heating and 90 minute cooling in 0.1M Citrate Buffer [49], 20 minute incubation in Pepsin (Sigma-Aldrich, St. Louis, MO) solution, and 20 minute incubation in 2N HCl (Sigma-Aldrich, St. Louis, MO) were performed. Endogenous peroxidase activity, avidin, and biotin were blocked with 30 minute incubations in 3% H_2O_2 and Streptavidin/Biotin solutions from a Blocking Kit (Vector, Burlingame, CA), respectively.

An overnight incubation in the primary antibody, CD71 Mouse Anti Rat (AbD Serotec, Raleigh, NC) at a 1:200 dilution, followed. Vector Laboratories' PK-7200 R.T.U. Vectastain Universal Elite ABC Kit (Vector, Burlingame, CA) was used as the secondary antibody source (at a 1:2 dilution). A 15 minute incubation in DAB (Vector, Burlingame, CA) was followed by a 1 minute incubation in the counterstain, Hematoxylin (Vector, Burlingame, CA). Slides were dehydrated in several dilution of ethanol before coverslips were applied with Paramount (Fisher Scientific, Pittsburgh, PA). One tissue section on each slide was reserved as a negative control, and was treated with Mouse IgG2a Negative Control (AbD Serotec, Raleigh, NC) instead of the primary antibody. Further, in each experiment one slide received neither the primary antibody nor the negative control to control for contamination in the primary antibody.

Results of immunohistochemical staining were assessed with microscope photography. Using SpotAdvanced software (Diagnostic Instruments, Sterling Heights, MI) pictures of villi were taken at 40x magnifications. Care was taken to photograph the same villus in the negative control tissue section and primary antibody treated section of each slide. Photographs were opened with Metamorph software (Molecular Devices, Sunnyvale, CA) and a region of approximately seven enterocytes was circled on both the experimental and corresponding negative control picture. A threshold for the brown color of DAB staining was defined for each slide. The percentage of area stained with DAB was quantified. The percent threshold of the negative control picture was subtracted from that of the experimental picture to yield the amount of TfR staining. Analysis was conducted for six villi on each slide. The average percent

threshold was calculated from the six replicates. At least five slides were analyzed for each experimental group.

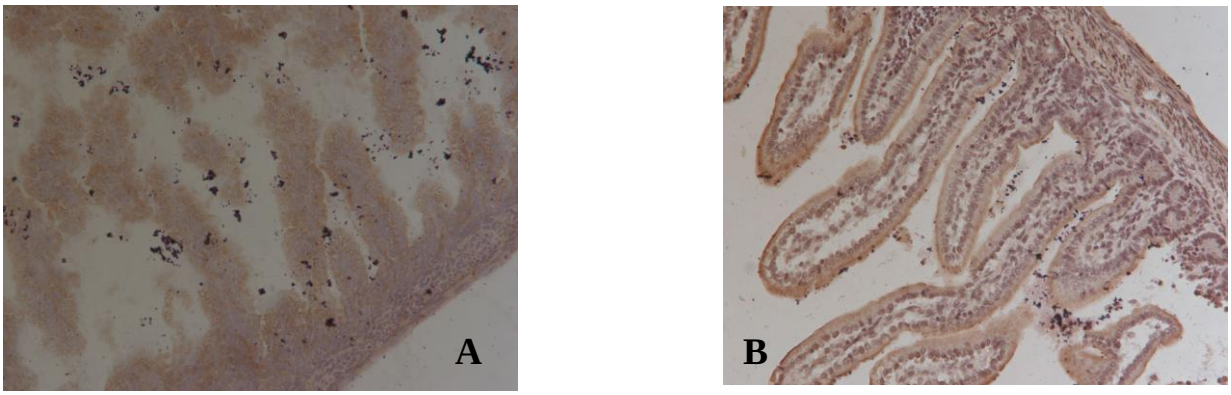

Figure 4: Preliminary immunohistochemistry results to compare frozen and paraffin preserved tissue. (A) Immunohistochemistry results for duodenum tissue of a 12 day old rat prepared on a frozen slide. Results are shown at 20X magnification. A great loss of tissue morphology was observed. (B) Immunohistochemistry results for duodenum tissue of a 12 day old rat prepared from paraffin preserved tissue, shown at 20X magnification. Methodology was preformed analogous to that of the slide in A, but tissue morphology is greatly preserved.

Normalization of Immunohistochemistry Results

Because TfR was expressed as a density, the results were corrected for differences in surface area among experimental groups. Using SpotAdvanced software (Diagnostic Instruments, Sterling Heights, MI) pictures of villi were taken at 10x magnification. Photographs were opened with Metamorph software (Molecular Devices, Sunnyvale, CA) and complete villi were circled. The area of each villi was calculated. At least 10 villus were analyzed from each experimental slide, and an average area was found. Area measurements were compared to results from Dam control, and the TfR expression (%) was subsequently corrected.

Prussian Blue Analysis

Slides containing Prussian blue stained liver tissue were photographed with SpotAdvanced software (Diagnostic Instruments, Sterling Heights, MI). For each liver slide, six central veins were located, centered in the view field, and photographed at 20x. Photographs were opened with Metamorph software (Molecular Devices, Sunnyvale, CA) and a threshold for the pink color characteristic of ferric iron/ferritin was defined using a positive iron control slide. The threshold of pink staining was quantified for each slide. The open area of the central vein was subtracted from the total area of the picture, and the remaining area was used to calculate the percent threshold of pink staining. The average percent threshold was calculated from the six replicates. At least 4 slides were analyzed from each experimental group.

H&E Analysis

Slides containing H&E stained liver tissue was analyzed analogously to Prussian Blue Analysis. Using Metamorph software, a threshold was created for the dark purple color characteristic of red blood cell pockets.

Normalization of Liver Staining Analysis

Prussian Blue and H&E liver staining was reported as a density. Because liver weight differed among groups, staining density was corrected for this difference. Duodenal weights

were compared to the average Dam control weight, and staining densities were subsequently corrected.

Statistics

Data were statistically analyzed by ANOVA, Fisher's PLSD tests. In the following figures, 'a' denotes statistical significance when compared to Dam, 'b' when compared with Dam/Epo, and 'c' when compared to IDA.

Results

Duodenal weights were greater in IDA (4.3±0.4) and IDA/Epo (3.8±1.0) than in Dam (2.6±0.2) or Dam/Epo(3.0±0.15). Addition of Epo did not statistically change weights (Figure 5). The corrected percent expression of TfR was significantly higher in IDA/Epo (31.159±5.521%) compared to Dam (17.372±1.726%). Dam/Epo (23.184±4.700%) and IDA (27.161±5.533%) did not differ significantly, however a nonsignificant trend of increasing expression with treatment was observed (Dam<Dam/Epo<IDA<IDA/Epo) (Figure 6). Representative results of immunohistochemistry staining are shown in Figure 7.

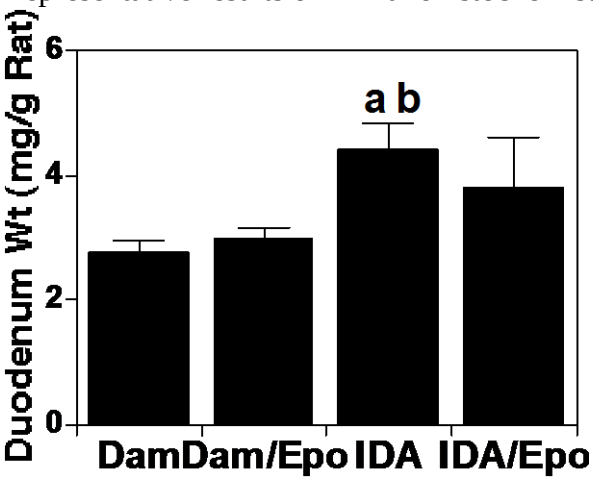

Figure 5: Average duodenal weights for experimental groups. Duodenums were measured to be statistically heavier in IDA compared to Dam (P=0.0042) and Dam/Epo (P=0.0147). Error bars denote a standard error of ±1.

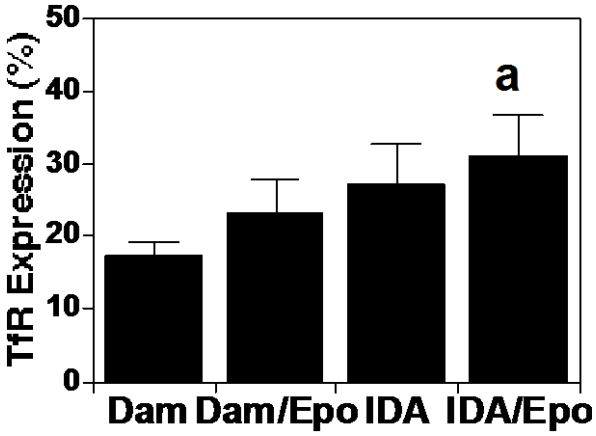

Figure 6: The percentage of enterocyte containing stain for TfR. The expressed percentages were corrected for differences in exposed surface area among experimental groups. A statistical difference was seen among expression in IDA/Epo compared to Dam (P=0.0420). Error bars denote ±1 standard error)

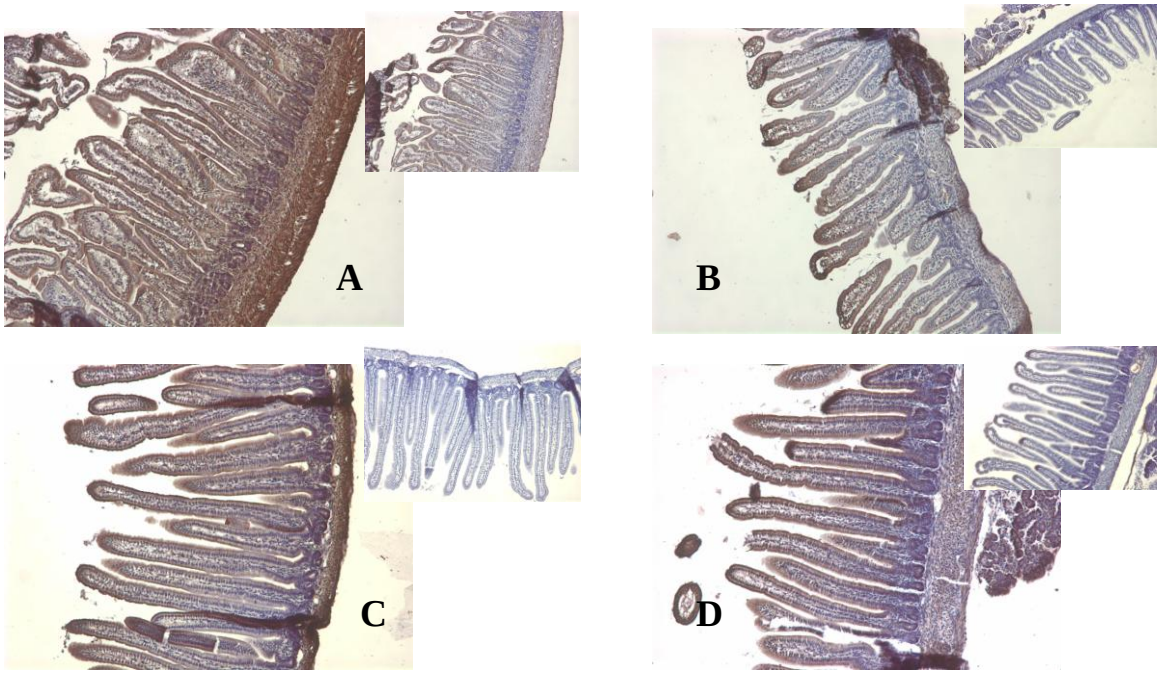

Figure 7: Results of immunohistochemistry DAB staining (brown) for TfR in rat duodenal tissue. Hematoxylin (purple) was used as a counterstain to locate cell nuclei. Large pictures depict staining of experimental tissue, while small pictures in the upper right show staining of the negative IgG2a control of the same tissue. Pictures where taken at 10x magnification of (A) Dam, (B) Dam/Epo, (C) IDA, and (D) IDA/Epo tissues.

No difference was observed in the total body weight between Dam (26.943±0.830 g), Dam/Epo (27.019±0.569 g), IDA (27.111±0.842 g) or IDA/Epo (27.333±0.712 g) (Figure 8). However, liver weights were greater in IDA (37±1 mg/g rat) and IDA+ Epo (35±2 mg/g rat) than either Dam (32±1 mg/g rat) or Dam/Epo (29±1 mg/g rat). Treatment with Epo did not statistically alter liver weight (Figure 9).

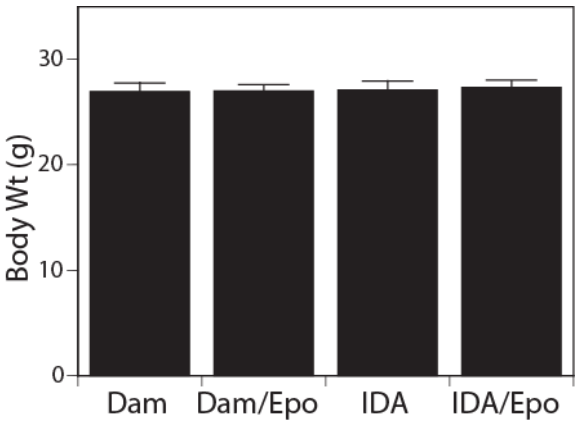

Figure 8: Body weight measurements for each experimental group. No statistical difference was seen among treatments. Error bars denote ±1 standard error.

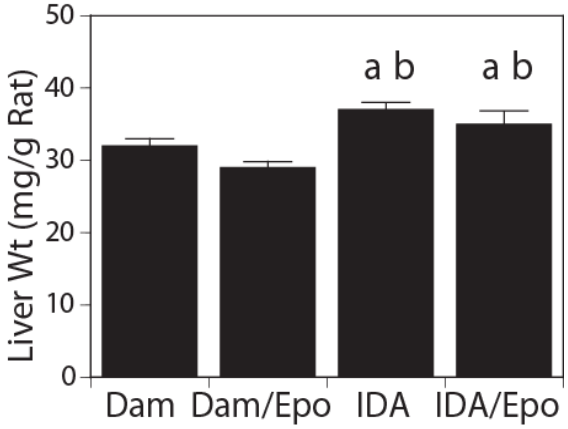

Figure 9: Liver weight measurements for each experimental group. IDA was statistically heavier than Dam (P=0.0016) and Dam/Epo (P<0.0001). IDA/Epo was statistically heavier than Dam (P=0.05) and Dam/Epo (P=0.001). Error bar denote ± 1 standard error.

We observed that feeding Epo to iron sufficient dam rats did not appear to increase body iron content (µg/g rat weight). We have not yet analyzed body iron from the IDA plus Epo rats (Figure 10). Here, liver iron was compared. Liver iron was greater in IDA (4.943±0.390 µg/g rat wt) and IDA/Epo (5.130±0.419 µg/g rat wt) than in Dam (2.198 ±0.585 µg/g rat wt) or Dam/Epo (1.644±0.452 µg/g rat wt) (Figure 11)

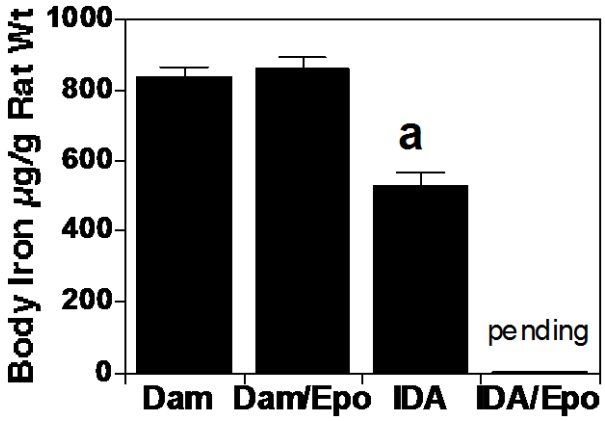

Figure 10: Body iron measurements for Dam, Dam/Epo and IDA. IDA/Epo results are pending. A statistical difference was seen between Dam and IDA (P<0.05). Error bars denote ±1 standard error.

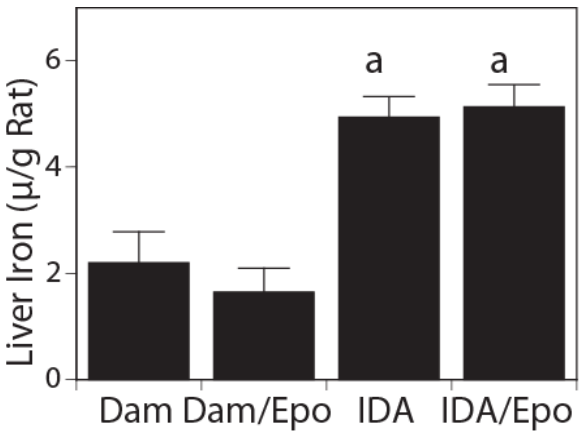

Figure 11: Liver iron measurements for each experimental group. A statistical difference is seen between IDA and Dam (P=0.0002) and IDA/Epo and Dam (P=0.0002). Error bars denote ±1 standard error.

Corrected Prussian blue staining of the liver to show ferric iron/ferritin revealed lower staining percentages in IDA (0.461±0.136%), IDA/Epo (0.294±0.064%), and Dam/Epo(0.553±0.060) compared to Dam (2.971±1.202%) (Figure 12). Representative staining pictures are shown in Figure 14. Corrected H&E staining for red blood cell pockets in liver

tissues were not statistically different among Dam (1.595±0.296%), Dam/Epo (0.736±0.199%), IDA (1.447±0.454), or IDA/Epo (1.440±0.758%). However, a trend of lower red blood cell pocket percentage was seen in Dam/Epo (Figure 13). Representative H&E staining pictures are shown in Figure 15.

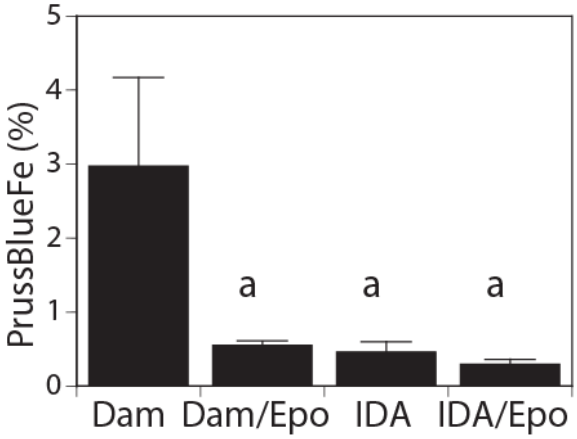

Figure 12: Prussian blue staining of the liver for ferric iron/ferritin. The reported percent staining was corrected for difference among liver weights in the four experimental groups. A statistical difference is seen between Dam and Dam/Epo (P=0.0027), IDA (P=0.0030), and IDA/Epo (P=0.0005). Error bars denote ±1 standard error.

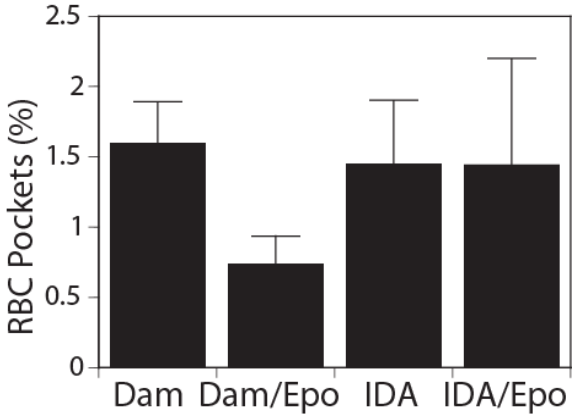

Figure 13: Liver H&E staining for red blood cell pockets. The reported percent staining was corrected for differences seen in liver weights. A statistical difference was not seen among experimental groups. Error bars denote ±1 standard error.

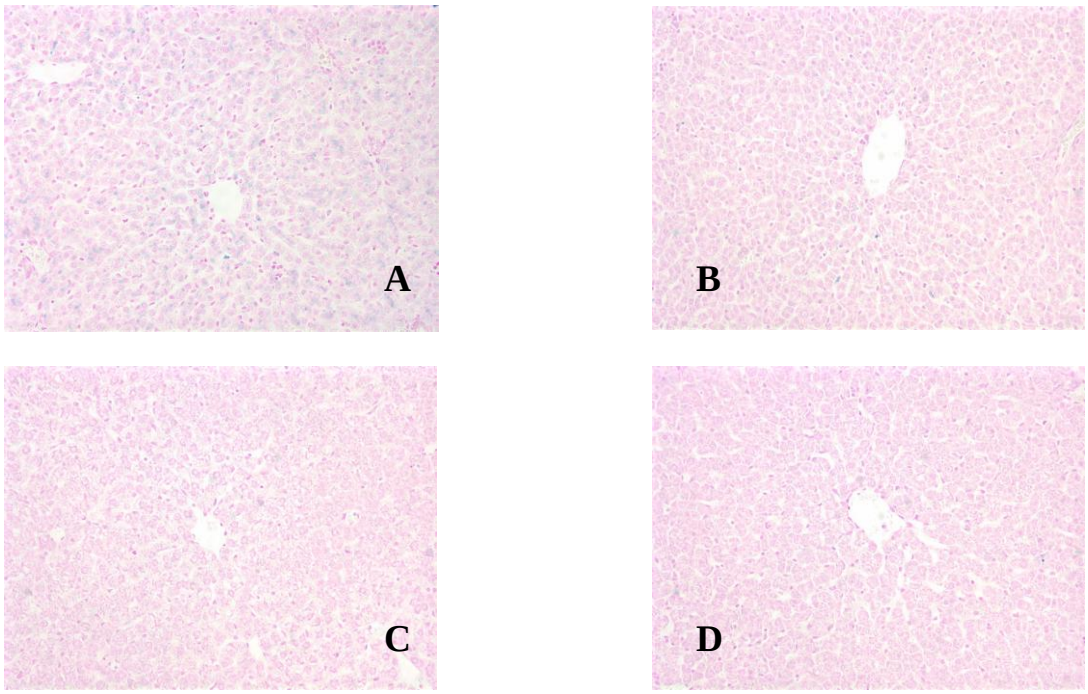

Figure 14: Prussian Blue staining of liver tissue at 20x magnification. Prussian Blue stains ferritin iron blue. Prominent staining is seen in (A) Dam tissue, but little staining is observed in (B) Dam/Epo, (C) IDA, or (D) IDA/Epo.

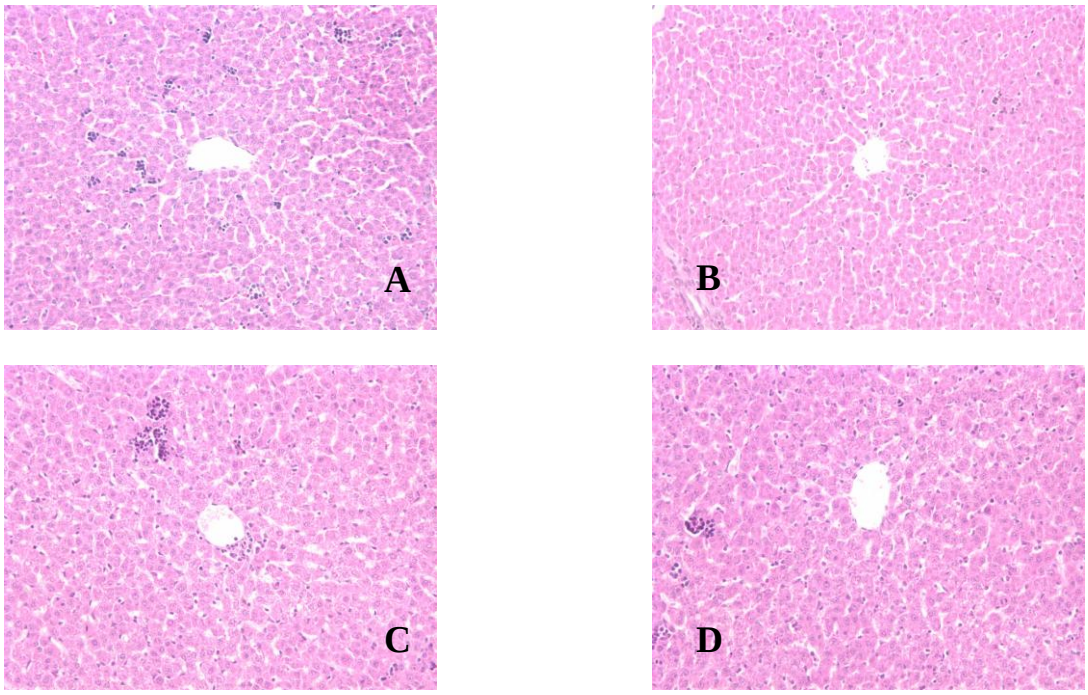

Figure 15: Results of H&E staining of liver tissue. Red blood cells pockets appear as dark purple granules. Pictures were taken at 20x magnification. Tissues from (A) Dam, (B) Dam/Epo, (C) IDA, and (D) IDA/Epo treated animals are shown.

In an attempt to explore the possibility of inflammation, white blood cell counts were compared across groups. The white blood cell count was higher in IDA (8.611±1.428 10^6/L) than in Dam (3.890±0.656 10^6/L), Dam/Epo (5.006±0.820 10^6/L), and IDA/Epo (4.660±0.489 10^6/L (Figure 16).

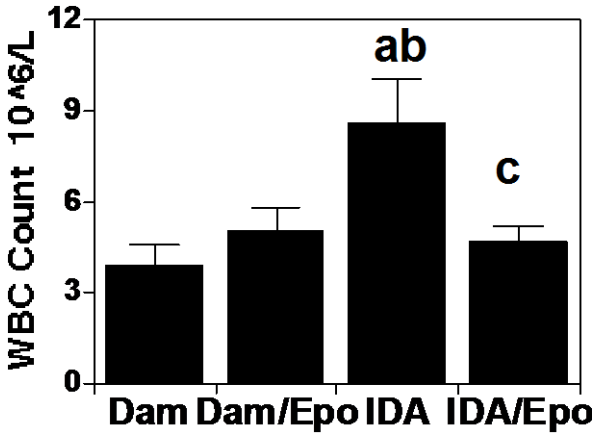

Figure 16: Average white blood cell count for experimental groups. IDA was significantly higher than Dam (P=0.0012), Dam/Epo (P=0.0124), and IDA/Epo (P=0.0197). Error bars denote ±1 standard error.

Conclusions

TfR Concentration

We hypothesized that TfR concentration would increase with both iron deficiency and the addition of Epo. Although insignificant, our results did trend in support of this hypothesis, as TfR concentration was higher in IDA than Dam, and Epo treatment increased TfR in both experimental groups. It will be necessary to increase the sample size of this analysis to determine if this trend is significant. Further, body iron analysis after Epo treatment will illustrate if iron absorption is increasing with increased TfR.

Several studies have supported the increase of TfR with increased erythropoiesis and iron deficiency [36]. Further, these results are consistent with the accepted mechanism of iron absorption, which cites regulation of TfR, DMT1 and ferritin levels by iron regulatory proteins [50]. However, Pinto et al did not observe significant changes in TfR mRNA levels among rats treated with recombinant Epo [51]. Thus, it will be necessary to increase sample size, or perform an immunoblot to determine if the trend observed in these data is significant.

Body, Liver, and Duodenal Weights

While body weights were constant across experimental groups, liver weights were higher in IDA than Dam. This increase in weight suggests increased storage of iron in the liver of rats fed IDA. In an attempt to characterize this increase in weight and iron, liver tissue was stained for ferritin (Prussian Blue) and red blood cell pockets (H&E). Surprisingly, ferric iron was highest in Dam livers, and remained constant among other treatment groups. Red blood cell pockets were reduced in Dam/Epo, but otherwise did not differ. Neither of these observations account for increased liver iron and weight accompanying IDA treatment. Dvorak et al. reported that increased growth of organs is common with artificial feeding in neonatal rats, even when body weights do not differ from Dam [48]. Increased liver weights seen here are consistent with this observation.

Similarly, duodenal weights measured heavier the IDA group. Both IDA and Epo are known to increase villus length [48,42]. Additionally, Juul et al observed a dose-dependent increase in villus surface area with enteral Epo treatment [39], suggesting that the body is

attempting to increase iron absorption by increasing the surface area available for absorption [22]. It is unclear why IDA promotes higher duodenal weights, although Dvorak et al. reported similar findings between IDA and Dam rats [48].

Inflammation & Hepcidin

We propose that inflammation accompanying IDA contributes to these trends. Balla et al. reported that pro-inflammatory cytokines are involved in regulation of several genes that effect iron uptake, storage, and utilization [50]. If inflammation accompanies IDA, the body could react as it does in infection, by retaining cellular iron through macrophage accumulation and interruption of duodenal iron absorption [25]. Increased inflammation could be a factor in increased liver and duodenal weights seen here with IDA.

In addition, cytokines involved in the inflammatory response are known to promote the production of hepcidin [50,52]. Hepcidin is a peptide mainly produced in the liver [52]. Hepcidin is a negative regulator of iron absorption, and as levels rise the small intestine down regulates ferroportin and DMT-1, and the release of iron from macrophages, which is controlled by ferroportin, becomes blocked [50,32,51,52]. Evolutionarily, this immune mechanism has evolved to inhibit bacteria infections and tumor development, which are generally accompanied by inflammation. Hepcidin is a defensin and iron is necessary for tumor growth and bacteria metabolism [52].

As a large population of macrophages are found in the liver [32], this sequestration may be a contributing factor in the increased liver iron observed in IDA rats. We tested for liver iron in the form of ferritin and red blood cell pockets, in an attempt to understand the increase of liver iron with IDA. Neither of these forms of iron were increased in IDA. Liver macrophages are a vital part of iron recycling in the body. Of the 25 mg of iron needed daily for erythropoiesis in human adults, only one to two mg are absorbed through the duodenum, while the rest is supplied by macrophages [25]. Liver macrophages phagocytose old and damaged red cells and recover hemoglobin iron. This iron is either stored within the macrophage or released to bind transferrin [25]. In the presence of heightened hepcidin, this release is repressed [32].

We expected that iron within macrophages would stain with Prussian blue, however results of staining did not support liver iron sequestration. Our method of quantitative staining analysis by color threshold may not have been sufficient to identify the more homogeneous blue staining produced by iron-laden macrophages. Guo et al. used a double staining method of Prussian Blue and α-smooth muscle actin to identify iron-containing macrophages [53]. This method of staining may be more useful in identifying if the observed liver iron increase in IDA is due to macrophage sequestration of iron. Further, Prussian Blue only stains Hemosiderin iron, so that it is possible that the iron in the vessels, in enzymes, or in ferritin form could account for the extra iron observed in IDA livers. It would be critical to do IHC for ferritin in the liver specimens. By using a negative stain for Prussian Blue and positive stain for ferritin, this theory could be assessed.

The hypothesis that inflammation is related to IDA is supported by white blood cell counts. As expected in inflammation, IDA rats had statistically higher white blood cell counts than Dam. This increase was not observed in IDA/Epo, supporting that Epo acts as an anti-inflammatory. Additionally, preliminary C-reactive protein (CRP) data from our lab shows higher levels in IDA than Dam, indicating inflammation. It will be important to access CRP levels of experimental groups treated with Epo, to see if the expected decrease in CRP with treatment is observed.

In addition to acting as an anti-inflammatory, increased Epo levels are also associated with decreased hepcidin expression. Erythropoiesis is associated with decreased hepcidin levels [32]. Parenteral Epo treatments have resulted in a significant decrease in liver hepcidin expression [52]. The work of Pinto et al. suggests that Epo plays a direct role in the regulation of hepcidin, as hepcidin mRNA levels decreased dose-dependently with Epo treatment, and mouse hepatocytes and human hepatoma cells produced less hepcidin *in vitro* with the addition of Epo [51]. Thus, Epo may play an additional function in facilitating iron absorption through the down regulation of hepcidin.

The pro-inflammatory cytokine IL-6 has been identified as an inducer of hepcidin expression [51,52]. IL-6 is one of several cytokines that is known to reduce TfR expression [25]. Thus if hepcidin expression in IDA is accompanied by IL-6 expression, TfR expression may be lower than we hypothesized. This may account for the insignificant difference observed in our TfR expression data. Further, hepcidin expression appears to decrease intestinal iron absorption regardless of iron status [52].

Necessary Future Studies

In order to better understand the role of TfR concentration in iron absorption, this experiment should be extended to include Dam and IDA rats treated with both Epo and ferrous sulfite. Our lab has previously shown that newborn rats fed enteral Epo and ferrous sulfate simultaneously have increased erythropoiesis [42]. Research by Skikne et al on adult patients with renal failure indicates that iron supplementation may be necessary to meet the increased erythropoietic needs accompanying Epo therapy [44]. Body iron and liver iron analysis of these additional experimental groups would allow us to correlate TfR concentration to changes in iron absorption. Further, immunohistochemistry or immunoblots for ferroportin and DMT-1 would also be helpful in determine the relationship between iron deficiency, Epo, and enterocyte iron transporters.

In addition, feeding radio labeled iron to animals in each experimental group would allow for observation of where iron concentrates after absorption [44,31]. If heightened hepcidin levels cause sequestration of Fe in the liver, we would expect to observe more labeled iron in the liver of IDA than Dam. If the Epo is able to down regulate hepcidin, we would expect to see lower labeled iron in the liver of IDA/Epo than IDA. ELISA analysis (DRG International Inc., Mountainside, NJ) of hepcidin expression in the four experimental groups would also increase understanding. Based on our hypothesis, we would expect higher hepcidin in IDA than Dam, and lower expression in IDA/Epo than IDA. Results from these studies would provide for a more thorough understanding of Epo's role as a regulator in both inflammation and iron absorption.

Finally, if erythropoietin is shown to affect iron levels our previous experiment on the effects of pasteurization of milk could be extended to this rat model. This would allow us to examine the effects of pasteurization a specific biologically active factor, erythropoietin, in an *in vivo* system. Using the same technique used to feed IDA rats, milk would be collected from a dam, pasteurized, and then fed to rat pups using a pup in the cup apparatus. If this experiment showed no difference in TfR levels of rats reared on pasteurized and unpasteurized milk, the results of our *in vitro* pasteurization experiment would be consistent with an *in vivo* model. Such findings would provide additional evidence that biologically active factors can withstand pasteurization without harm. This would provide further support for the use of pasteurized milk from milk banks as an optimal nutritional food source for premature infants.

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