# Assessing the Pathological Effect of Maternal Malnourishment and Fetuin-B on Placental Tissues

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# Introduction

## Overview

Low birth weight is a prevalent issue in many countries throughout the world. In 2017, 8.28% of babies born in the U.S. were born with a low birth weight (LBW), meaning they had a birthweight of less than 2,500 grams (CDC, 2017). Globally, in 2015, almost 15% of all births (20.8 million) were characterized as LBW (UNICEF, 2015). LBW can be caused by a number of factors including maternal undernutrition (MUN) and placental dysfunction. While there is a fair amount of information regarding the impact of undernutrition on LBW, not much is known about the specific biological factors/proteins that lead to placental dysfunction that causes LBW. Previous research found that the concentration level of the protein fetuin-B may be increased in a malnourished mother and then accumulate in the fetal body. Once in the fetus, fetuin-B negatively impacts fetal development by inhibiting organ formation. The goal of the current study was to examine the effects of fetuin-B on the placenta in order to determine how fetuin-b passes through the placental barrier and to evaluate its role as a negative factor in placental health and development.

## Review of Literature

**Intrauterine growth restriction and the placenta.** Low birth weight (LBW) is a common issue and has many potential causes. One such cause is maternal undernutrition (MUN) (Gabbey, 2019). In addition, LBW can be caused by intrauterine growth restriction (IUGR) (Pagano, 2018). IUGR is characterized as suboptimal growth in the fetus which can lead to impairment and underdevelopment of fetal organs.

Previous research has revealed that the placenta experiences dysfunction in the IUGR model. The placenta aids in fetal development by serving as an interface between maternal

circulation and fetal circulation. It allows for the exchange of nutrients, dissolved gases, and wastes between the mother and the fetus without physically mixing the blood from each. Trophoblasts, which are placental cells, are integral parts of placental morphology. Trophoblasts are a diverse group of placental cells that allow the placenta to carry out many of its major functions, including allowing nutrient transport between the mother and the fetus. Trophoblasts help to anchor the embryo to the uterus, which is a process that is known as implantation. Along with implantation, trophoblasts also help to form vascular connections between the maternal circulation and the fetal circulation that allow for the exchange of essential nutrients and wastes between the fetus and the mother (Cross et al., 1994). Cytotrophoblasts, or the undifferentiated form of trophoblasts, differentiate into different subsets, each with specific morphologies and functions. One of these subsets is the extravillous trophoblast. Extravillous trophoblasts work to anchor the villi to the placental wall (Huppertz et al., 2008). The current study focuses on extravillous trophoblasts because they help to anchor the placenta to the mother and are thus a vital point of connection between the fetus and the mother.

Previous research has examined the differentiation of trophoblast progenitors, or precursor cells, in response to trophoblast death. The growth, death, and differentiation of human cytotrophoblasts within villi were examined. The cytotrophoblasts were taken from first trimester human placentas that were obtained after termination of pregnancy. A major finding was the behavior of the extravillous cytotrophoblast. The extravillous trophoblast progenitors that were located at the tips of the villi remained viable and capable of proliferation and subsequent outgrowth in order to become extravillous trophoblasts. (James, et al., 2005). This study showed that the ability for extravillous trophoblasts to proliferate is an important part of their function. In

the current study, extravillous trophoblasts were examined for proliferation in order to determine how fetuin-B impacts the function of trophoblasts.

**Low Birth Weight and Renal Health.** Previous research has shown that maternal undernutrition (MUN) can lead to decreased organ development, which is a symptom of intrauterine growth restriction (IUGR), in low birth weight (LBW) mice (Barnett, et al., 2017). In this research, the kidneys experienced decreased organ development in LBW fetuses. A decrease in blood flow in the kidneys was indicated by a 37% decrease in renal blood perfusion. A decrease in the number of functional units in the kidneys was shown by the 69% decrease in total glomerular volume. These indicate a decrease in kidney development by showing a decrease in renal function and structural development. While the effects of LBW on the murine neonate were known, the health of the LBW adult was unknown.

In order to evaluate the implications of LBW later in life, the health of LBW mice as they grew older was also examined. It was found that as LBW mice progressed in age, both male and female mice experienced a decrease in renal and cardiovascular function. A decrease in the health of the kidneys as time went on was indicated by a decrease in renal blood perfusion, which means that there was a decrease in the rate at which blood flows through the kidneys (Fig. 1). Nitrosative stress was indicated by elevated levels of reactive nitrogen species (RNS) in the kidneys. Nitrosative stress occurs when too much RNS is present in the body. RNS are a type of highly reactive molecule derived from nitric oxide-descended free radicals. Free radicals are molecules that have an unpaired electron, making them very reactive (Fig. 2). RNS cause nitrosative stress which can

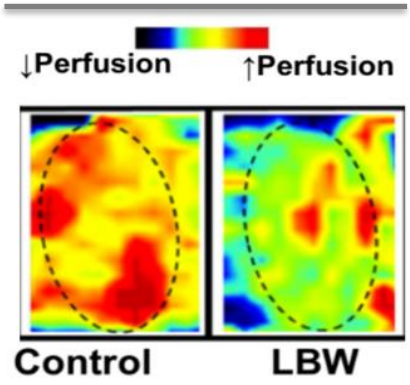


**Figure 1:** Representative image of decreased blood perfusion in the LBW model in 6-month old male mice (Abdulmahdi *et al.*,

severely damage tissues (Abdulmahdi et al., 2018). This particular study established that the impacts of LBW were drastic, severe, and not significantly improved as time went on. Now that the effects of LBW were known, especially its adverse effects on the kidneys, the focus of research shifted to determining a specific factor that could cause this damage to the LBW fetal kidney.

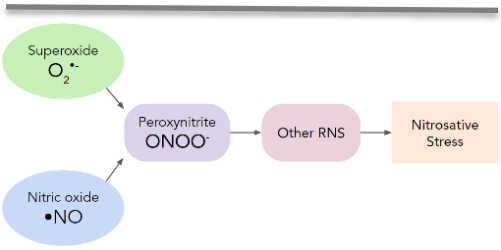


**Figure 2:** flowchart depicting how RNS are formed and how they lead to nitrosative stress (Figure by A. Rath)

Barnett et al. also found that there was a decrease in the overall quantity and quality of renal stem cells ($SIX2^+$ cells) in LBW pups. An overall decrease in kidney function and development in the LBW pups was a result of a 76% decrease in $SIX2^+$ stem cells. While it was clear that renal stem cells and progenitors were experiencing cell death and apoptosis, it was unclear which specific factors contributed to this.

**Fetuin-B in the Fetal Kidneys.** Later research identified the protein fetuin-B as a potential factor that impeded kidney development in the LBW neonate that was born as a result of MUN. In cell culture studies, it was found that fetuin-B led to increased apoptosis and cell death of renal stem cells ($SIX2^+$ cells) by increasing oxidative stress (Fig. 3). Oxidative stress, is similar to nitrosative stress, but it activates different biological pathways. Oxidative stress occurs when there is an increase in reactive oxygen species (ROS), which are highly reactive compounds that are of an oxygen lineage. It was found that fetuin-B caused the apoptosis of $SIX2^+$ cells and inflammation was caused by the NFkB

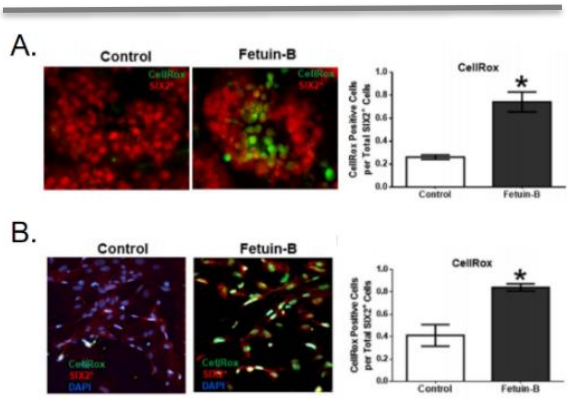


**Figure 3:** Fetuin-B treatments increased levels of ROS, as shown in embryonic kidney explant staining (A) and primary tissue culture staining (B). (Rabadi *et al.*, 2018)

pathway, which is influenced by ROS. The NFkB pathway is heavily influenced by ROS, so the elevated levels of ROS caused by fetuin-B likely led to NFkB-induced inflammation (Fig. 4). There is strong support for the idea that the increase in inflammation that was caused by NFkB could have led to the apoptosis of the $SIX2^{+}$ cells.

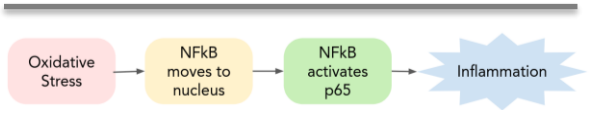


**Figure 4:** Flowchart depicting the process by which oxidative stress causes NFkB-induced inflammation by activating p65, which is located in the nucleus of the cell (Figure by A. Rath)

Renal stem cells were also stained for proliferation, or the growth and spread of cells. Elevated levels of fetuin-B inhibited the function of renal stem cells by decreasing their ability to proliferate, which was shown by the fact that fewer renal stem cells stained for proliferation (Rabadi et al., 2018).

In addition, Rabadi et al. studied the location of upregulated fetuin-B. Fetuin-B's source was not primarily the fetus, despite the fact that most of its deleterious effects were observed in the fetal body. While the fetuin-B caused major damage to the LBW fetus and renal development, its source was not primarily the fetus. It was found that in the MUN model, the vast majority of fetuin-B that was in the fetus came from the mother, passed through the placenta, and then accumulated at higher levels in the fetus. Once in the fetal circulation, the fetuin-B caused severe damage to renal stem cell quality and quantity. While it was known that fetuin-B crossed the placental barrier to get from the maternal circulation to the fetal circulation, it was unknown how fetuin-B was able to cross the placental barrier. Fetuin-B was shown to increase oxidative stress and apoptosis while also decreasing the proliferation of renal stem cells. However, fetuin-B's effects on placental cells (trophoblasts) are unknown. Knowing the effects of fetuin-B on trophoblasts will reveal the mechanism of how fetuin-B crosses the placental barrier and will thus lend more insight into fetuin-B's role in placental and fetal development.

## Problem Statements

1. In the maternal undernutrition-induced model of low birth weight, the effects of elevated fetuin-B in the fetal kidneys is known. However, the effects of elevated fetuin-B in the malnourished mother's placenta is unknown.
2. While the effects of fetuin-B specifically on the renal stem cell are known, the effects of fetuin-B on the trophoblast are unknown.
    a. The specific effects of fetuin-B on the proliferation, oxidative/nitrosative stress, inflammation, and apoptosis on trophoblasts are unknown.
    b. The concentration of RNS in the MUN placental tissue/trophoblast are unknown.

## Objectives

1. To examine the effects of elevated fetuin-B within the murine malnourished mother's placenta by performing immunohistochemistry staining for fetuin-B and oxidative stress in order to determine if maternal malnourishment causes fetuin-B and oxidative stress to be increased in the placenta.
2. To examine the effects of elevated fetuin-B on the human trophoblast in order to examine the effects of fetuin-B specifically on the trophoblast.
    a. To examine the effects of fetuin-b on the human trophoblast using immunocytochemistry staining for oxidative stress, nitrosative stress, proliferation, inflammation, and apoptosis in order to evaluate the effects of fetuin-B on the human trophoblast.

b. To examine the effects of MUN on the amount of nitrosative stress in the murine placenta by performing Western Blot in order to evaluate if nitrosative stress is present in the MUN trophoblasts.

## Hypotheses

1. It was hypothesized that the malnourished murine mother will have elevated fetuin-B and increased oxidative stress in the placenta. Previous research has shown that fetuin-B was increased in the MUN maternal circulation, and that elevated fetuin-B increased oxidative stress in the fetal kidneys.
2. It was hypothesized that elevated fetuin-B will decrease the health and quality of trophoblasts by inducing oxidative and nitrosative stress.
    a. It was hypothesized that fetuin-B will increase oxidative stress, nitrosative stress, inflammation, apoptosis, and decrease proliferation, in human trophoblasts after conducting immunocytochemistry. Previous research has shown that fetuin-B has had similar effects within renal stem cells. Therefore, it was expected to see mirrored results within the trophoblast.
    b. It was hypothesized that fetuin-B will increase nitrosative stress, as previous research has established that RNS and nitrosative stress has led to the degeneration of kidneys. Similarly, it is expected to see that placental degeneration caused by fetuin-b will be partially due to elevated levels of RNS.

## Methodology

### Role of student vs. mentor

Over the course of eight weeks during the past summer, I participated in immunohistochemistry staining for placental explants, immunocytochemistry staining for

placental trophoblasts, BCA protein assay, western blot, cell quantification, and analysis of data. The supervising scientist conducted the placenta sectioning in preparation for immunohistochemistry, the portions of the cell culture procedure that involved live cells, TUNEL staining for immunocytochemistry, as well as providing training and materials needed for the procedures. The student conducted immunocytochemistry staining for the presence of reactive oxygen species (ROS), reactive nitrogen species (RNS), trophoblast proliferation, trophoblast inflammation, and trophoblast apoptosis. The student also conducted Western Blot for the presence of RNS, and quantified immunohistochemistry, immunocytochemistry, and Western Blot.

**Immunohistochemistry**

Immunohistochemistry was performed in order to examine the effects of elevated fetuin-B as a result of maternal malnourishment on placental tissue. Placental studies were performed in order to examine the levels of fetuin-B and ROS that aggregate within the living animal. In accordance with the IACUC # 18-2-0618, the supervising scientist placed pregnant mice on an MUN diet consisting of low protein, low calorie chow, and placed controls on a diet with normal chow; this would establish the experimental group of MUN mothers that served as a basis for the murine model of the study. The supervising scientist sacrificed the pregnant mice at E17 ($17^{th}$ day of pregnancy) and excised the murine placentas. The supervising scientist then cryosectioned the placentas in order to get tissue samples to be used for staining. Then, the supervising scientist placentas were stained with CellROX and for the presence of fetuin-B. Fetuin-B staining was used in order to see how much fetuin-B was located in the trophoblasts. In order to assess the level of oxidative stress present, CellROX staining, which stained for reactive oxygen species (ROS), was used. Immunohistochemistry staining allowed for the analysis of fetuin-B's effects

on the placenta, but immunocytochemistry needed to be performed in order to examine fetuin-B's specific effects on the trophoblast.

**Immunocytochemistry**

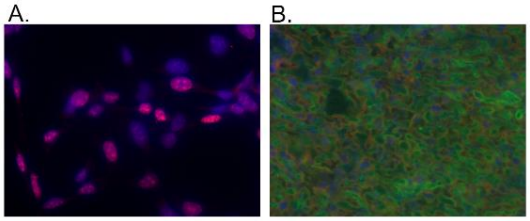


**Figure 5**: Representative image showing the morphological differences of individual trophoblast cells (A) and placental tissue (B). Trophoblast cells are used to determine effects of fetuin-B on trophoblasts specifically. (Image by A. Rath)

Immunocytochemistry was performed in order to examine the effects of elevated fetuin-B on placental trophoblasts specifically (Fig. 5). The human trophoblast HTR-8/SVneo cell line, purchased from the American Tissue Culture Collection, was used for immunocytochemistry. Immunocytochemistry was performed in order to examine levels of oxidative stress due to reactive oxygen species and reactive nitrogen species, proliferation, and apoptosis. In order to establish the experimental model that would test the effects of fetuin-B on the trophoblast, the experimental groups were treated with recombinant fetuin-B while the control groups were treated with serum-free medium. In order to determine the amount of oxidative stress, CellROX was used to stain for ROS. In order to determine the amount of nitrosative stress, NOX4 was used to stain for RNS. In order to assess the effects of fetuin-B on the trophoblast's ability to proliferate, Ki67 was used to stain for proliferation. In order to assess the amount of trophoblast cell death, TUNEL was used to stain for apoptosis. In order to determine the potential apoptotic pathway that was induced by fetuin-B, NFkB p65 staining was conducted. The presence of NFkB-p65 staining within the nucleus indicated the progression of the NFkB p65 inflammatory pathway. In short, cells at 75% confluency were transferred to chamber slides containing fetal bovine serum (FBS) medium and incubated at 37℃ for 24 h. Then, the experimental group was treated with fetuin-B and incubated for 24 h at 37℃. The control group was treated with serum-free medium. Then, the cells were washed with

PBS and fixed with 4% PFA in PBS for 30 min. at room temperature, then washed again. Next, in order to permeabilize them to prepare for staining, cells were treated with 2.5% BSA in PBS-Triton X 0.1% for 10 min., then washed. Cells were then blocked for 1 h at room temperature with 5% PBS-BSA. Then, the cells were treated with primary antibodies and incubated overnight at 4℃. The primary antibodies were specific to the target molecule, but they did not fluoresce. In order to have the stains fluoresce, a fluorescent secondary antibody was used. After washing, the cells were treated with the secondary antibody and incubated for 1 h at room temperature. Then, the cells were washed again, the chambers were removed, DAPI fluoromount was added, and coverslips were sealed with nail polish. It was expected to see an increase in oxidative stress staining, a decrease in proliferation, an increase in nuclear NFkB staining, and an increase in apoptosis in the MUN model.

**Western Blot**

**BCA Assay.** A BCA Assay was performed in preparation for Western Blot. Specifically, the BCA assay was done to determine the loading volumes per sample, per well for Western Blot. Sections were taken from frozen murine placentas, both controls and MUN placentas, and digested in a solution consisting of 6 mL RIPA buffer and 1 mL protease inhibitor, with 500 µL being added per sample. Sections were homogenized with a biovortexer and vortexed to mix in order to ensure that the protein within the sample was evenly distributed. Then in order to separate the supernatant from the pellet, samples were centrifuged for 10 mins. at 10,000 rpm. In order to improve the accuracy of the assay, the supernatant for each sample was diluted 1:1 with RIPA buffer. Then, 3 µL of each supernatant mixture was pipetted into a 96-well plate. In order to help calibrate the assay, baseline concentrations were also added to the well plate. Next, 200 µL of a 1:25 dilution of A:B BCA reagents were pipetted into each well. The well was then

incubated at 37°C for 20 min., then run through a plate reader. The concentration of protein per sample was determined, and loading volumes were calculated so that the same amount of protein would be loaded per well.

**NOX4 Analysis.** Western Blot was performed in order to verify a result of immunocytochemistry. In immunocytochemistry, when assessing for oxidative stress as a result of reactive nitrogen species (RNS), it was found that there were no significantly elevated levels of NOX4 staining in the MUN placentas. Western Blot was performed in order to verify this lack of positive staining. In order to denature the protein, 75 µL of each sample used in the BCA Assay was diluted with 25 µL Laemmli buffer. Samples were then boiled for 5 min., then vortexed and centrifuged. Using the volumes determined in the BCA assay, each sample was loaded into the wells in the gel. In order to provide a scale for the rest of the measurements, 10 µL of ladder was also added into a well. The gel was run at 100 V for 1 hr to separate the bands of protein, and then the gel was removed from the casing. At this point, the protein is not visible on the gel, so in order to be able to stain the protein, the protein on the gel needed to be transferred to a membrane (Fig. 6, A) . Before performing the transfer, in order to avoid potential electrical fire, the membrane was polarized with methanol. Then, in order to prepare for the transfer, the sandwich containing the gel and the membrane was assembled and inserted into the cassette, submerged in cold transfer buffer, and run for another 1 hr (Fig. 6, B). After transfer, in order to decrease the possibility of false positive staining, membranes were blocked in a 5% milk solution

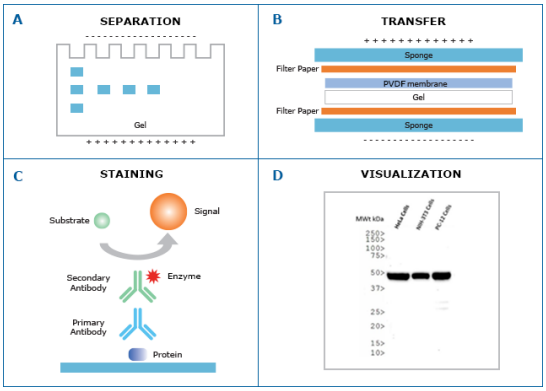


**Figure 6:** the general steps of western blot. Specific proteins are separated into bands (A), the protein bands from the gel are transferred to a membrane (B), the membrane is stained for the target protein (C), and the protein bands are visualized (D). (Novusbio.com)

and incubated at room temperature for 1 h. Next, after rinsing with rinse buffer, the membranes were treated with the NOX4 primary antibody and incubated overnight at 4℃. Then, the membranes were washed and incubated for 1 h at room temperature with the NOX4 secondary antibody, which is fluorescent. Then, in order to enhance the intensity of the fluorescence to make the results easier to quantify, the membranes were incubated with a 1:1 solution of Super ECL solution for 5 min at room temperature while covered (Fig. 6, C). Next, the membranes were imaged for NOX4 in the Doc-It$^2$ 315 Imager (Fig. 6, D). After imaging the NOX4, in order to establish a baseline to measure the NOX4 against, β-actin needed to be imaged. After imaging NOX4, in order to remove the NOX4 antibodies, the membranes were stripped with stripping solution and washed with wash buffer. Then, β-actin primary antibody was incubated with the membranes at 4℃ overnight. Then, after washing, the membranes were treated with the β-actin secondary antibody for 1 h at room temperature. After incubation in the 1:1 Super ECL mixture for 5 min., the membranes were again imaged in the Doc-It$^2$ 315 Imager. β-actin was used to establish a baseline stain for the intensity of the bands.

**Data Quantification**

Image J was used for analysis of data. For staining, integrated density was calculated for NOX4 and fetuin-B. Integrated density is a measure of the intensity and "brightness" of a certain stain. Positive staining over total cells was counted for CellROX, Ki67, and TUNEL staining (Fig. 4). Positive staining was determined by counting the total number of cells that stained positively for the stain target and dividing this number by the total number of cells (shown by blue DAPI staining). Western Blot was also quantified using Image J, with the intensity of the bands for NOX4 being compared to the bands for β-actin.

**Statistical Analysis**

Graphpad Prism was used in order to generate graphs based on the data calculated and acquired using Image J. The statistical test used was Student's T-test, which is used to compare two groups against each other and determine the statistical significance of the experimental group. Since each procedure (immunohistochemistry, immunocytochemistry, and Western blot) compared two groups (experimental vs. control), statistical significance was determined by using Student's T-test. Since immunohistochemistry and Western blot used murine placenta samples, the MUN group was being compared to the control group. Immunocytochemistry utilized cell culture, so the cells treated with fetuin-B were tested against the control group.

## Results and Discussion

### Immunohistochemistry

In placental staining for fetuin-B, there was a significant increase in placental fetuin-B in the MUN mice ($p < 0.05$; Fig. 7). This increase in fetuin-B shows that elevated levels of fetuin-B are located in the placentas of malnourished mothers when compared with controls. Therefore, the basis of this study, that fetuin-B that is located placenta, was validated. Staining for reactive oxygen species, denoted by CellROX staining, increased significantly in the MUN model as well ($p < 0.01$; Fig. 8). This shows that the placental trophoblasts, or placental cells, of the malnourished mothers experienced more oxidative stress as a result of reactive oxygen species compared

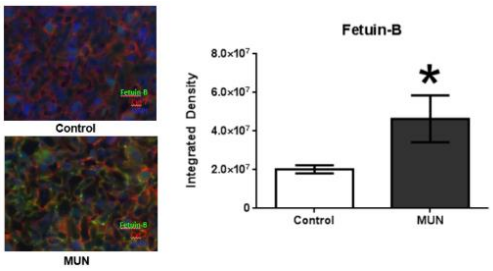


**Figure 7**: comparison of intensity of Fetuin-B staining (green) between MUN and Controls – Tissue Studies. Fetuin-B was significantly increased in the MUN placenta. ($p < 0.05$, N=3-5) (Figure by A. Rath)

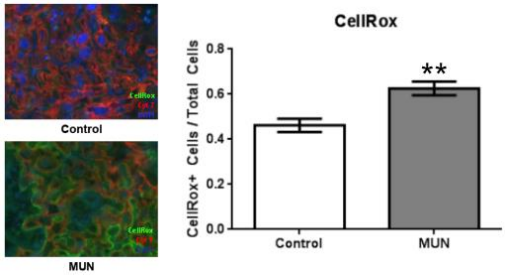


**Figure 8**: comparison of intensity of CellROX (ROS) staining between MUN and Controls – Tissue Studies. ROS levels were significantly increased in the MUN placenta. ($p < 0.01$, N=5) (Figure by A. Rath)

to their control counterparts. Taken together, these results confirm that the placentas in the MUN model experienced more oxidative stress, and that this elevated oxidative stress was most likely due to the elevated levels of fetuin-B present in the malnourished placenta. This elevated oxidative stress is indicative of the mode of damage of fetuin-B. Increased oxidative stress caused by fetuin-B can activate inflammatory pathways and subsequent apoptotic pathways. These results mimic the harmful effects of fetuin-B that were observed within the fetal kidneys.

**Immunocytochemistry**

In human placental trophoblasts, staining for proliferation, reactive nitrogen species (RNS), reactive oxygen species (ROS), inflammation, and apoptosis was conducted in order to determine the type of stress that was caused to the trophoblast, and how the function of the trophoblast was impacted by fetuin-B. A decrease in trophoblast proliferation (shown by the Ki67 stain) was expected because previous research had shown a decrease in proliferation in renal stem cells treated with fetuin-B. This was confirmed, as the fetuin-B treated cells experienced a significant decrease in proliferation compared to their control counterparts ($p < 0.0001$; Fig. 9). Trophoblasts were also examined for presence of free radicals

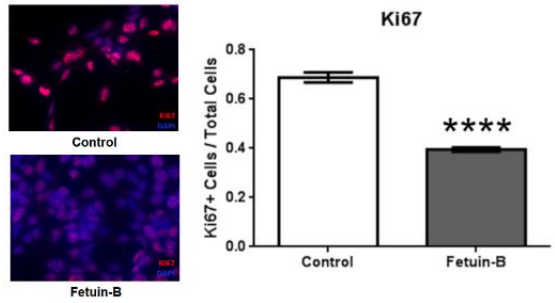


**Figure 9**: comparison of intensity of Ki67 (proliferation) staining between fetuin-B and Controls – Cell Culture. Proliferation was significantly decreased in the cells treated with fetuin-B ($p < 0.01$, N=4) (Figure by A. Rath)

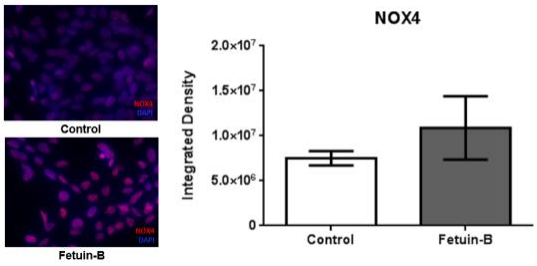


**Figure 10**: comparison of intensity of NOX4 (RNS) staining between fetuin-B and Controls – Cell Culture. There was no significant difference in the levels of ROS in the cells treated with fetuin-B. (Figure by A. Rath)

(ROS/RNS), indicating oxidative and nitrosative stress. There was no significant increase in NOX4 staining in the fetuin-B trophoblasts, indicating that there was no significant increase in reactive nitrogen species or subsequent nitrosative stress ($p > 0.05$; Fig. 10). This showed that the source of damage was not due to stress that was a result of free radicals are not nitrosative in origin. However, a significant increase in CellROX staining was observed in the fetuin-B trophoblasts, indicating elevated levels of reactive oxygen species and elevated oxidative stress, showing that the stress caused to the cells was due to free radicals that are of an oxygen lineage ($p < 0.01$; Fig. 11). These results indicate that fetuin-B leads to elevated oxidative stress and decreased proliferation in the human trophoblast, showing a decrease in function of the trophoblast (shown by the decreased proliferation), and an increase in stress to the trophoblast (indicated by increased oxidative stress). Since fetuin-B does not significantly elevate RNS, it can be inferred that the damage caused to the human trophoblasts was a result of the increase in ROS.

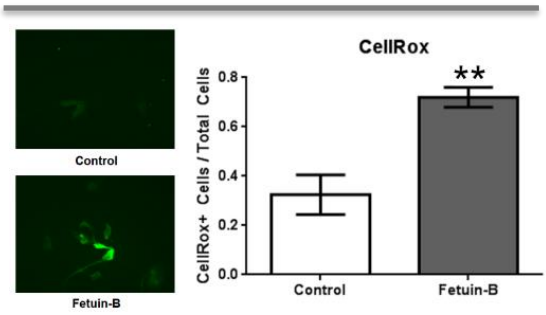


**Figure 11**: comparison of intensity of CellROX (ROS) staining between fetuin-B and Controls – Cell Culture. There was a significant increase of ROS in the cells treated with fetuin-B ($p < 0.01$, N=4) (Figure by A. Rath)

There was also a significant increase in the staining for NFkB in the nucleus within cells treated with fetuin-B ($p < 0.05$; Fig. 12). This increase in the staining of nuclear NFkB indicates the progression of the NFkB inflammatory pathway. The increase in inflammation indicates the effects of elevated ROS within the trophoblasts, because the NFkB pathway is

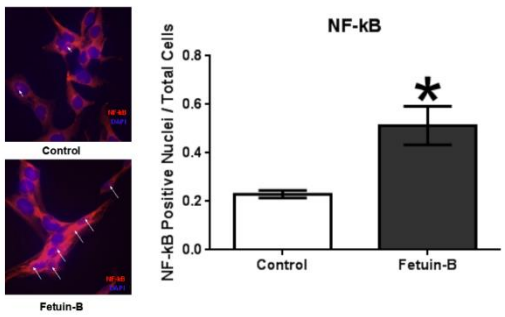


**Figure 12:** Comparison of intensity of NFkB (inflammation) staining between fetuin-B and Controls – Cell Culture. There was a significant increase in nuclear NFkB staining (as shown by the white arrows) in cells treated with fetuin-B (N=4) ($p < 0.05$) (Image by A.

heavily influenced by the presence of ROS. Trophoblasts treated with fetuin-B also exhibited a significant increase in apoptosis, as indicated by the increase in TUNEL staining ($p < 0.05$; Fig. 13). This apoptosis can also be caused by the increase in oxidative stress seen within the trophoblasts, as previous research had found that oxidative stress can cause cell death by way of apoptosis (Ryter et al., 2006). This increase in apoptosis of trophoblast cells, along with the increase in inflammation, show that the trophoblast was negatively impacted by fetuin-B. These results indicate that fetuin-B contributed to the progression of trophoblast cell death by increasing inflammation and by increasing apoptosis.

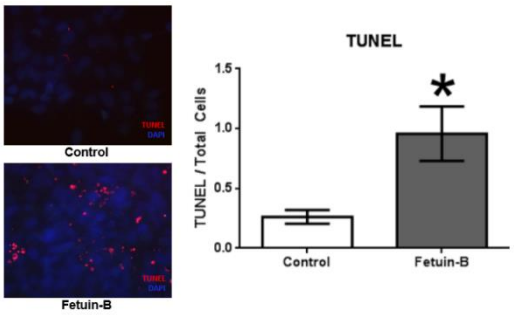


**Figure 13:** Comparison of intensity of TUNEL (apoptosis) staining between fetuin-B and Controls – Cell Culture. There was a significant increase in TUNEL staining of DNA fragments in trophoblasts treated with fetuin-B ($p < 0.05$). (Image by A. Rath)

**Western Blot**

Western Blot analysis was conducted in order to confirm the result of immunocytochemistry displaying no significant increase in NOX4 staining and denoting the amount of RNS present in the tissue. When Western Blot was conducted using samples from MUN and control murine placentas, a similar result was observed: there was no significant increase in the levels of NOX4 in the MUN placenta ($p>0.05$; Fig. 14). This confirms the finding in the ICC study that fetuin-B does not lead to an increase in RNS in trophoblasts. Since it has already been shown that fetuin-B is examined at elevated levels within the MUN placenta, and that fetuin-B does not lead to an increase in RNS and nitrosative stress, with these findings it

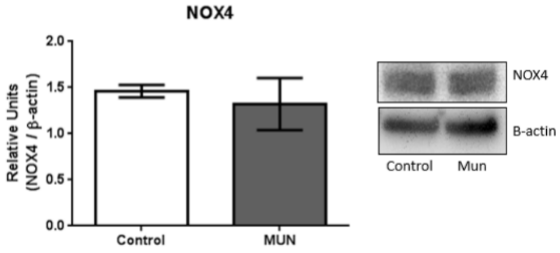


**Figure 14**: Comparison of intensity of NOX4 staining between MUN and Control: Western Blot. There was no significant increase in NOX4 in the MUN placenta. (N=5) (Figure by A. Rath)

can be seen that elevated fetuin-B as a result of the MUN model did not lead to elevated RNS and nitrosative stress, either.

When examining the findings from IHC, ICC, and Western Blot together, various conclusions can be drawn. It was hypothesized that elevated levels of fetuin-B as a result of maternal malnourishment negatively impacts trophoblast quantity and quality and placenta quality due to oxidative stress, however the null is rejected in favor of the hypothesis that fetuin-B has deleterious effects on placental cells and tissues, especially by elevating oxidative stress. The placental staining established the validity of the experiment by demonstrating the there was elevated placental fetuin-B in the MUN mother. The CellROX staining also indicated that there was increased ROS and oxidative stress within the MUN placenta. The ICC studies using human trophoblasts confirmed that the results found in the placentas were due to fetuin-B and not a different factor. The ICC also confirmed that the elevated oxidative stress led to a decrease in trophoblast quality, as shown by the decrease in proliferation (Fig. 9). Healthy trophoblasts are supposed to experience a relatively high level of proliferation, but this was inhibited by fetuin-B treatment. Finally, Western Blot was performed in order to validify the results of the ICC, that RNS and nitrosative stress were not results of elevated fetuin-B. These results were confirmed from the lack of positive staining after running western blot (fig. 14). Not only did this confirm that nitrosative stress is not present as a result of fetuin-B, it also demonstrated that it is not a result of this model of maternal malnourishment.

It has been established that reactive oxygen species and oxidative stress have worked to promote the progression of renal injury (Ratliff et al., 2018). Later research determined that low birth weight mouse pups experienced a decrease in kidney function and formation (Barnett et al., 2017). Then, it was found that oxidative stress promoted kidney injury in the LBW murine adult

(Abdulmahdi et al., 2018). In another study, it was found that the protein fetuin-B induced damage to renal stem cells and inhibited proper kidney formation in the LBW neonate (Rabadi et al., 2018). Similar to Rabadi in 2018, the effects of MUN-induced Fetuin-B on various tissues was examined. However, unlike the previous study, the effects of fetuin-B on the placenta were examined. Rabadi in 2018 had found that maternal fetuin-B was somehow able to cross the placental barrier into fetal circulation, where it then damaged fetal kidneys as renal stem cells by inducing oxidative stress. It was then hypothesized that fetuin-B was able to cross the placental barrier by implementing a similar mode of damage to the placenta and placental trophoblasts. The results of this study indicate that fetuin-B's deleterious effects are much more far reaching than originally hypothesized in 2018, as it negatively impacts different types of tissues in a similar way. This research shows that fetuin-B's negative effects are multi-dimensional: not only does it inhibit kidney formation in the fetus, it also damages the placenta, thus promoting suboptimal fetal growth.

**Applications**

The placenta is an enigmatic organ, and research into its function and role within pregnancy is still ongoing. Research into the function of the placenta and its role in development is important as it can reveal potential therapeutic targets in the context of placental damage. This research, along with previous research, has uncovered fetuin-B as a factor that has severely negative effects on the placenta and subsequent fetal development. Along with revealing information regarding the function of the placenta, this research also uncovered more functions of fetuin-B that were previously unknown. Not much is known about the function of fetuin-B compared to other proteins within the fetuin family, but this research exposed another role of fetuin-B in the process of fetal development and pregnancy.

The results from this study stress the importance of proper maternal nutrition during pregnancy. It is important for expecting mothers to eat a healthy, balanced diet including all essential macronutrients and micronutrients. It also emphasized the importance of consuming enough antioxidants to limit the amount of oxidative stress that occurs within the placenta. It is recommended that the average pregnant mother consumes around 80-85 mg of vitamin C (which is an antioxidant) daily during pregnancy (American Pregnancy, 2019). These antioxidants help to protect tissues against damage, thus also helping protect the developing fetus by decreasing the potential for impaired organ formation. With proper nutrition and the intake of adequate antioxidants, it is possible that an expecting mother can minimize the potential for suboptimal growth of her baby by negating some of the negative effects of fetuin-B. These effects of fetuin-B are damaging and life-threatening to the unborn fetus and neonate; however, they can be mediated by carefully controlling and regulating the diet of the mother.

## Future Research

Future studies can focus on determining other sources of oxidative stress in order to gain a better understanding of the types of reactive oxygen species (ROS) that fetuin-B induces. This would reveal more potential therapeutic target that can be used in future studies. After observing that nitrosative stress is not a major symptom of elevated fetuin-B, it is possible that other reactive oxygen species are of a mitochondrial lineage. Future research can include mitosox staining for mitochondrial superoxide dismutase. This would reveal any potential ROS that come from the mitochondria, and thus potentially reveal another source of more ROS. Future research can also focus on treatments for the oxidative stress. Since fetuin-B is still a relatively mysterious protein and currently there are no specific inhibitors for the protein, treatments can target stresses that are caused by fetuin-B, like oxidative stress, with the aim to ameliorate the symptoms of the

stress. Since the free radicals that cause the oxidative stress are of an oxygen lineage, future studies can focus on giving pregnant malnourished mothers an ROS-specific antioxidant supplement. A popular antioxidant is superoxide dismutase (SOD), which acts as a catalyst for the dismutation of superoxide into oxygen and hydrogen peroxide (Ratliff et al., 2018). Artificial forms of SOD, such as tempol, have shown promising results in ameliorating symptoms in previous studies. Tempol was shown to decrease circulating levels of ROS within mice that were born LBW, as well as improve mortality in males that were born LBW (Abdulmahdi, et al., 2018). Since antioxidant treatment has shown positive results in the past in terms of improving kidney health, it has the potential to improve placenta health.

Other future research can be conducted to determine the levels of apoptosis that are induced by fetuin-B and if they considerably increase the levels of apoptosis that are caused by hypoxia/reoxygenation. Hypoxia/reoxygenation happens during ischemia-reperfusion, which is a mode of damage to the placenta that occurs during preeclampsia. Preeclampsia is a potentially life-threatening injury that can be caused by poor maternal nutrition, which is similar to how fetuin-B is upregulated within the pregnant mother. Hypoxia-reoxygenation occurs during ischemia-reperfusion, which happens when a large influx of oxygenated blood damages placental tissues after an extended period of low oxygen. During healthy placental development, it is essential that there is a period of low oxygen, or hypoxia, in order to ensure proper trophoblast differentiation. However, previous research had found that within the model of preeclampsia and ischemia-reperfusion, the period of reoxygenation that occurs after hypoxia can lead to the apoptosis of trophoblasts (Hung, 2002). Previous research has also determined that hypoxia/reoxygenation was a possible source of oxidative stress during preeclampsia (Hung, 2006). Therefore, future research can also focus on determining the amount of apoptosis and

oxidative stress caused by fetuin-B compared to the levels observed in preeclampsia. This is because preeclampsia (and subsequent hypoxia/reoxygenation) occurs in similar conditions that are said to increase levels of fetuin-B: poor maternal nutrition. Determining the levels of fetuin-B's oxidative stress and apoptosis would provide a more holistic view of how damaging fetuin-B is to the function of the entire placenta compared to other modes of damage.

## Conclusion

The objective of this study was to examine the role of elevated fetuin-B and reactive oxygen species on the placenta and placental trophoblasts in order to determine fetuin-B's role in placental development. To evaluate this objective, immunohistochemistry staining of murine placentas, immunocytochemistry of human trophoblasts, and western blot were conducted. Staining showed that MUN leads to elevated fetuin-B and ROS, decreased proliferation, and that there is no effect on nitrosative stress. These results indicate a process by which fetuin be causes damage within the MUN model: the MUN model leads to elevated fetuin-B within the placenta; this elevated fetuin-B then leads to the aggregation of ROS in trophoblasts and placental tissue; this elevated ROS level leads to oxidative stress; and this oxidative stress leads to a degeneration in the overall health and function of the placenta, especially by reducing proliferation of trophoblast cells. This study has demonstrated and stressed the need for proper nutrition during pregnancy. With this research, we are one step closer to truly understanding the impacts of malnutrition on the function of the placenta, and how the placenta helps to dictate the health of the child..

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