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Research Plan

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

Rationale

Low birth weight is a prevalent problem throughout the world, as 8.28% of babies born in the US in 2017 were born low birth weight, or LBW (CDC, 2017). LBW neonates are described as babies born at a weight of less than 2,500 grams, or about 5 pounds 8 ounces. This is usually a result of improper fetal development. The placenta is an organ that is necessary in proper fetal development. It is located within the mother during gestation and allows for the transfer of nutrients, gases, and wastes between maternal circulation and fetal circulation without physically mixing the maternal blood and the fetal blood. The placenta does this through villi. Villi are placental structures that float in a cavity of free-flowing maternal blood. The villi themselves contain fetal capillaries, which are surrounded by mesenchymal stroma, along with trophoblasts. Trophoblasts are placental cells that take on different phenotypes. The individual cytotrophoblasts surround the layer of mesenchymal stroma, while the multinucleated, continuous layer of fused trophoblasts is the syncytiotrophoblast. The syncytiotrophoblast surrounds the cytotrophoblast monolayer. Finally, extravillous trophoblasts anchor the tips of the villi to the placental wall (James et al., 2005). These trophoblasts are all essential to the proper function of the placenta, but the current study will focus on the extravillous trophoblast. LBW is a very prevalent problem throughout the world, but not much is known about the intricacies of the role of the placenta in fetal development. Thus, it is necessary to identify the different factors that play a role in the LBW model. Previous research in the LBW model was conducted within the kidneys. Previous research has found that while many organs were negatively impacted in the

LBW neonate, the kidneys were especially underdeveloped. At the end of kidney formation, there was a 32% reduction in the number of glomeruli and nephrons (Barnett et al., 2017) Subsequent research then examined the effects of the LBW model on kidney function as the mice progressed in age. It was found that there was a decrease in overall kidney function as time went on, as well as diminished overall glomerular capacity and decreased renal blood perfusion. Both male and female mice experienced a 55% reduction in glomerular filtration rate, as well as a 30% decrease in renal blood perfusion (Abdulmahdi et at., 2018). Then, research was focused on determining a specific factor that led to damage caused to fetal kidneys. The protein fetuin-B was identified as a factor that caused this damage by inducing oxidative stress. Oxidative stress is due to high levels of reactive oxygen species, or ROS, that react with other biological compounds and inhibit their function. Phenotypically, oxidative stress can appear within tissues as increased inflammation, degradation, and apoptosis of cells (Ratliff et al., 2016). It was found that increased levels of fetuin-B led to an increase in oxidative stress and subsequent degeneration and apoptosis of renal stem cells ($SIX2^+$ cells) on a massive scale, thus leading to the underdevelopment of fetal kidneys. When fetuin-B was administered to healthy pregnant mothers, the embryos experienced a 50% decrease in stem cell count, as well as a subsequent 66% decrease in nephron count (Rabadi et al., 2018). Interestingly, however, fetuin-B was not upregulated within the fetus itself. Instead, it was upregulated within the mother, crossed the placental barrier, and aggregated in the fetus where it then damaged renal stem cells. His-tagged fetuin-B that was injected into the mother was shown to be present within the fetus 30 minutes later (Rabadi et al., 2018). Thus, this study will be focused on determining how fetuin-B is able to cross the placental barrier. Specifically, the effects of fetuin-B on extravillous trophoblasts

will be examined, with an emphasis on fetuin-B's ability to induce oxidative stress within the trophoblasts.

Research Question

- How does fetuin-B that is upregulated as a result of MUN impact the pathology of placental tissue, especially trophoblasts?

Hypothesis

- If MUN-induced fetuin-B and oxidative stress upregulation are found within the placenta, then it is expected to find a decrease in trophoblast quality and quantity. Previous research has revealed the negative effects of fetuin-B and the subsequent oxidative stress that it causes on renal stem cells, so evidence suggests that fetuin-B could have a similar effect on trophoblasts.

Expected Outcomes

- It is expected to find that the increased levels of fetuin-B located in the blood of MUN mice will lead to oxidative stress within the placenta. Specifically, it is expected to find increased levels of the reactive oxygen species NADPH oxidase (NOX4). It is expected to find increased levels of oxidative stress because previous studies have found that increased levels of fetuin-B have led to increased oxidative stress and oxidative stress-induced apoptosis in renal stem cells as well as a decrease in overall fetal kidney formation and function (Rabadi et al., 2018). Thus, it is expected to see mirrored results within the trophoblasts and placenta. This would indicate that fetuin-B crosses the placental barrier by damaging placental trophoblasts, especially by increasing oxidative stress to levels that inhibit the function of these trophoblasts.

Procedure

Role of Mentor

During experimentation, the mentor will provide materials such as pipettes, the solutions needed for staining (PBS, blocking solution), the antibodies needed for staining (primary and secondary antibodies for Ki67 and HLA-G), the placental tissue samples from mice, and the microscope. The mentor will receive samples of the murine placental tissues from the animal department. The placental tissues will come from pregnant healthy controls and mice that have been placed on the MUN diet. The mentor and supervisor will provide training for proper laboratory techniques, such as pipetting, and protocols, such as immunohistochemistry staining. The mentor and supervisor will also conduct cell culture passages under the biological safety cabinet, as well as any treatments that are done to live cells. Using these materials and information, the student will be the primary generator of results for this section of the larger overall project. All handling of animals, including treatment and sacrifice, will be conducted by the mentor in collaboration with qualified personnel within the animal housing facility at New York Medical College. The student will not be in contact with live animals.

Role of Student: Experimentation

Imaging for Murine Placental Sections

- Imaging at 40x magnification, will take 4 images of different fields per placenta section
- DAPI (blue) and T_x (red) fields will be used to capture DAPI staining and positive antibody staining. Individual fields and merged image will be taken
- Pictures will be analyzed using Image J. Specifically, cells positively stained will be counted, integrated density and area will be measured.

Human Trophoblast Tissue Cultures: Staining for ROS

- All work involving the live cells and any work requiring the use of a biological safety cabinet will be done by the mentor.
- Confluent cells (75-90%) will be transferred to chamber slides containing FBS medium and let incubate at 37℃ and 5.0% CO_2 for 24 h
- Cells will be washed and treated with 16 µl fetuin-B per well, diluted with serum-free medium, and let incubate for 24 hours at 37℃ and 5.0% CO_2
- After incubation, cells will then be treated and stained for CellRox to asses for oxidative stress and damage, NFkB-p65 to asses for inflammation, and Cytokeratin 7 will be used as a trophoblast marker.
- Cells will be washed with PBS, then fixed with 4% PFA in PBS for 30 min. at room temperature, then washed 3-5 times with PBS
- Cells will be treated with 2.5% BSA in PBS-Triton X 0.1% for 10 min (150 µl per cell), then washed 2-3 times with PBS.
- Cells will be blocked for 1 hr at room temperature with 5% PBS-BSA, 150 µl per cell
- The primary antibody will be diluted (1:50-1:500) in 2.5 % blocking solution and incubated overnight at 4℃
- The cells will then be washed 3-5 times for 5 minutes with PBS, then the secondary antibody will be added (1:500 dilution) and incubated for 1 hr (100 µl per well) at room temperature
- The cells will be washed 3-5 times with PBS, and then the chambers will be removed
- DAPI flouromount will bee added, coverslips will be added, and slides will be sealed.

BCA Assay: Preparation for Western Blot

- Will prepare solution of 6 mL RIPA buffer and 1 mL protease inhibitor to help digest tissue
- Will pipette 500 µl solution into labelled Eppendorf tubes (MUN and Control)
- Frozen mouse placentas (kept on ice) will be cut using scalpel and small piece will be placed in corresponding Eppendorf tube containing RIPA buffer and protease inhibitor solution using forceps
- Sections will be homogenized with biovortexer and mixed with vortex
- Samples in RIPA and protease inhibitor will be centrifuged for 10 mins. at 10,000 rpm
- Supernatant will be pipetted into fresh labelled tubes, and stored in -80℃ freezer; pellets will be disposed of as biological waste
- Supernatant will be thawed and diluted with RIPA buffer (1:1)
- 3 µl of each supernatant mixture will be pipetted into a well in a 96-well plate
- 200 µl of 1:25 dilution of A:B BCA reagents will be pipetted into each well
- The well will be incubated at 37℃, 5.0% CO_2 for 20 min., then run through a plate reader
    - Measurements gathered from plate reader will be used to determine loading volumes
- 75 µl of supernatant dilution will be added to new labelled Eppendorf tubes, and 25 µl of laemmli buffer will be added to each new tube. The tubes will be stored in a -80℃ freezer.

Western Blot

- Samples will be boiled for 5 min. at 100℃, then vortexed and centrifuged
- Appropriate amounts of sample calculated from BCA assay will be loaded into respective wells, including 10 µl of ladder

- The gel will be run at 100 V for 1 hr, and then the gel will be removed from the casing
- An appropriate size of membrane will be cut and polarized with methanol
- The sandwich will be assembled (2 white sponges, filter paper, gel, membrane, filter paper, white sponge), and inserted into the cassette that will be submerged in cold transfer buffer; this will be run for 1 hr at 100 V.
- Membranes will be blocked in a 5% milk solution and incubated at room temperature for 1 hr on a rocker
- The membranes will be rinsed with wash buffer and incubated with a 1:500 dilution of the NOX4 primary antibody and incubated overnight at 4℃ on a rocker
- The membranes will be washed 3x 10 mins. and incubated for 1 hr at room temperature with the NOX4 secondary antibody (1:5000).
- The membranes will be incubated for 5 mins. at room temperature in a dark environment with a 1:1 ratio of a Super ECL mixture
- The membranes will be imaged in the Doc-It^2 315 Imager
- The membranes will be stripped with stripping solution and washed with wash buffer.
- The β-actin primary antibody (1:20,000) will be incubated with the membranes at 4℃ overnight on a rocker
- After washing, the β-actin secondary antibody (1:5000) will be incubated with the membranes for 1 hr at room temperature on a rocker
- After incubation in the 1:1 Super ECL mixture for 5 min., the membranes will be imaged in the same manner as previously described.

Risk and Safety

- The laboratory will undergo routine risk assessment and safety inspections by New York Medical College EHS. The studies and experiment that the student will be conducting have been designated as BSL-1, as the student will be working with tissues and chemicals that do not require a high level of biological containment.
- The student will handle materials such as phosphate buffer solution (PBS), blocking solution, permeabilization solution, antibody solutions, mounting solution, nail polish, and placental sections. The placental sections are needed as they are the portions of the mice that the student will be examining using immunohistochemistry. All the other solutions are also necessary in the staining process. PBS will be needed to wash the tissue, blocking solution will be used to reduce noise within the image, permeabilization solution will be used to permeabilize the tissue, antibody solutions will be used to stain the tissue, mounting solution will be used to mount the coverslips, and nail polish will be used to seal the coverslips.
- The materials involved with trophoblast cell culture include growth medium to promote the growth of trophoblast cells, serum-free medium to inhibit the growth of trophoblast cells, test tubes, supernatant gathered after the cells will be isolated within a pellet, pipette tips, and wells.
- Materials such as pipette tips, test tubes, Eppendorf tubes, and materials that have come in contact with biological agents will be disposed of in red biological waste bins and routinely collected by New York Medical College EHS.

Data Analysis

- Images taken of staining will be quantified using Image J. The stained cells will be counted using Image J, and integrated density of staining will be quantified. Statistical testing will be Student's T-test when appropriate.

Potentially Hazardous Biological Agents Research

- *Source and biosafety level*
    - In this study, placental tissues will be used. Placental tissues will be taken from pregnant controls as well as mice that are on a modified diet in order to induce maternal malnourishment. Placentas will be frozen, sectioned, and placed on slides by the mentor. The student did not complete any sectioning or handle the mice directly.
    - Since the placenta samples are not infectious or contagious, it was assessed that they would be in BSL-1. The student will complete research in BSL-1, which is the lowest level of biological containment, as the student will mostly be pipetting relatively non-hazardous chemicals and compounds.
- *Animal Model*
    - The animals used will be pregnant mice that are either controls or mice put on the MUN diet. The MUN diet consists of decreased protein and decreased calories.
    - Pregnant mice will be used as they are a reasonable substitute for humans within the context of the research. Humans will not be used, as the experiment requires the pregnant mother to be malnourished. This would seriously, negatively, and irreparably damage the health of the unborn baby in humans. Mice will be used instead of a non-vertebrate organism because potential results involving non-vertebrate organisms will be difficult to interpret as they relate to humans. A

murine model is an acceptable model that will yield results that can be related to humans without physically endangering or harming the lives or well-beings of humans.

  - The sample size for the experiment will be about 10-20 mice. The number of mice will be enough to achieve statistical significance of results without implementing the MUN diet on too many animals.
  - The student will not handle any live animals, and will not implement the MUN diet on the mice. The animals will be housed in the animal facilities at NYMC, and the student will use placental tissues taken from these mice in the project.

- *Tissue Cultures*
  - Human placental trophoblast cultures will be treated with fetuin-B and then stained for ROS to asses for oxidative stress. Tissue cultures will be used to assess for the impacts of fetuin-B in human cells.
- *Safety precautions*
  - To ensure safety of everyone within the workspace, personal protective equipment such as gloves, lab coats, close-toed shoes, and long pants will be worn.
  - To ensure safety, placenta samples and treated cell cultures will be disposed of as biological waste, within biological waste disposal containers, as directed by New York Medical College Environment, Health, and Safety department.
  - To ensure safety, procedures involving exposure of tissue cultures will be conducted within the biological safety cabinet.

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