NASA WVSGC RIG

October 2020 Progress Report

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Project Title: Impact of *In Utero* Thyroid-Related Mechanisms on Development of Craniosynostosis Evaluated by Avian Skull Morphogenesis

Progress Report:

The proposed project for the NASA WVSGC Research Initiation Grant began on June 1st at the beginning of West Liberty University's Summer Undergraduate Research Experience (SURE) program. The SURE program was able to provide a stipend for one undergraduate student (Hannah Ankrom) to participate in summer research and my awarded NASA RIG was able to fund an additional undergraduate student (Carson Corrick) and the research project. The overall objective of the project was to enhance the knowledge about *in utero* thyroid-related mechanisms and how they relate to the development of craniosynostosis (CS). To meet this objective, the proposal had 2 aims: Aim 1 - 3D morphometric analysis of cranial sutural growth and morphology in chick embryos following in utero TH exposure; and Aim 2 - Determining the gene expression patterns in sutural tissue between developing flat bones of the skull of chick embryos following in utero TH exposure. For Aim 1, following collection of chicken embryos with induced thyrotoxicosis, embryos were to be taken to WVU Cancer Institute, Animal Models and Imaging Facility (AMIF) to collect high-resolution micro-CT images of the embryonic skulls using their SkyScan 1272. However, due to the unforeseen pandemic, and new social distancing guidelines, we were unable to use the facility to collect these images. Therefore, we began the project with Aim 2.

Majority of our work so far has been to establish a reliable model for testing Aim 2. We ordered fertilized chicken eggs (Charles River) and separated them into two groups: (1) controls – saline-injected and (2) experimental – thyroid-hormone-injected fertilized chicken eggs. The dose we decided on was 1µg T_4 + 0.5µg T_3 in a volume of 1µL per embryo, with equivalent volumes of saline in our control group. This dose was based on published methods by Darras VM (2019). Injections were planned to take place every other day beginning on embryonic day 4 (E4). The thyroid hormones were prepared according to manufacturer instructions (Sigma Aldrich). This involved dissolving the hormones (in solid form) in ammonium hydroxide and diluted to the desired concentration with phosphate buffered saline (PBS). The saline controls therefore contained complimentary volumes of ammonium hydroxide and PBS as the corresponding carrier solution. Following the first day of injection, all chicken embryos were nonviable within 12 hours of injections. It was hypothesized that the carrier solution was toxic for the chicken embryos since both saline and TH-injected groups died. Following troubleshooting with the manufacturer, it was determined that the ammonium hydroxide was toxic and that we could alternatively use sodium hydroxide to dissolve the hormones, and 0.9% NaCl to dilute to our desired concentration. A second batch of fertilized chicken embryos was ordered, and new injection solutions were made to repeat the study. In our second round we injected the fertilized chicken eggs, every other day beginning on E4. By E8, all of the TH-injected embryos were nonviable, while all of the saline-injected embryos were still viable (see Trial 1 in the following table). Therefore, it was concluded that the embryos were not able to develop with our originally proposed model of thyrotoxicosis. This led

to a series of questions regarding the cause of TH-induced death in these chicken embryos. This study was carried out for our saline-injected control embryos and we were able to collect samples for these embryos as planned at E19. To compare TH-related effects on the overall development of the embryos, we wanted to collect multiple samples that could be used in the comparison to those injected with TH (given a successful model). Therefore, in addition to sutural membranes (originally proposed), we also collected blood serum, livers and hearts. Since the liver and heart is also a target of TH, we decided that they would be helpful in determining widespread impacts of thyrotoxicosis.

Tissues collected from the saline-injected control embryos were used to establish the protocol for qRT-PCR analysis. Sutural membranes, hearts, and livers were homogenized and digested in TRIzol reagent. RNA was extracted using spin column-based kits (RNeasy Quiagen). First strand cDNA was created using the SuperScript™ III Platinum™ One-Step qRT-PCR Kit (Invitrogen) and then qRT-PCR was performed using custom probe and primer sets (Life Technologies) for both *FGFR1* and *TRα*. Expression of both of these genes are expected to increase with augmented TH activity. We analyzed expression levels from our saline-injected embryos to determine the levels of expression that will eventually be compared to a set of tissues/organs from TH-injected embryos. We found that *FGFR1* and *TRα* are expressed at ambient levels (compared to our house keeping gene *SDHA*) in both the heart and liver. Students presented this methodology as a pilot study at the virtual WVU SURE Symposium at the end of the summer. Their posters were titled “The Methodology of Determining *TRα* Expression in Avian Embryos Following Induced Maternal Hyperthyroidism” and “Inducing Maternal Hyperthyroidism in Chick Embryos to Study Involvement in the *FGFR1* Pathway.” During SURE, a new graduate student (Andrea Dwiggins) joined our lab to assist with our project into the fall 2020 semester.

It was unforeseen, but a part of starting a new research project, we had to overcome many challenges in working to establish a model for *in utero* thyrotoxicosis. The viability data that we collected from each attempt (we termed as “Trials”), has been extremely critical for getting closer to establishing this model. In conducting a more thorough investigation of the literature, we discovered that there is not a successful model involving sequential TH-injections with chicken embryos. Therefore, we had to consult studies that had marginal success and compare those to our results to design alternatives with the objective of developing an effective, and reliable model. A great deal of troubleshooting was executed, but the students were involved in every aspect and had the experience of both hands-on research skills and the critical thinking skills that engaged them throughout the entire process. To summarize the design of these trials, the table below lists the experimental design, rationale, and outcome for each trial.

Table 1: Overview of the experimental process establishing a reliable model of thyrotoxicosis

Trial	Design	Rationale	Outcome
Trial 1	As described above. Group A: N=11 saline-injected Group B: N=11 TH-injected (0.5μg T_3 + 1.0μg T_4) every other day from E4-E18	Aimed to have sequential injections of TH to optimize skeletal growth effects using the same dose as Darras VM (2019) who showed TH effects on avian development.	Group A: N=10 saline-injected embryos viable at E19 (91%) and tissues collected for controls Group B: N=11 TH-injected nonviable by E8
Trial 2	Group A: N=7 TH-injected (0.5μg T_3 + 1.0μg T_4) E10, E14, and E18 Group B: N=7 TH-injected (0.5μg T_3 + 1.0μg T_4) E14 and E18 Group C: N=7 TH-injected (0.5μg T_3 + 1.0μg T_4) E18	After declines viability with Trial 1 prior to E8, we decided to inject later in development when the thyroid gland is known to be functional (E10). This trial was to test out various injections points during later development to determine embryo viability when using the same proposed dose (0.5μg T_3 + 1.0μg T_4) with multiple, versus a single injection	Group A: N=7 TH-injected embryos nonviable by E15 (30% viable after 1st injection at E10; 0% viable after 2nd injection at E14) Group B: N=7 TH-injected embryos nonviable by E18 (0% viable before 2nd injection) Group C: N=6 TH-injected embryos viable (24-hours after single injection)
Trial 3	Group A: N=6 TH-injected (0.125μg T_3 + 0.25μg T_4) E12, E14, E16, and E18 Group B: N=7 TH-injected (0.15μg T_3 + 0.35μg T_4) E12, E15, and E18	While dose was decided on based on published results by Darras VM (2019), Darras had injected once before collected embryos 24-hours later. While Group C from Trial 2 survived one injection, we are aiming to for sequential injections. Therefore, one group received 1/4th the total dose over 4 injections days and another group received 1/3rd the total dose over 3 injections days, to still have an overall dose of 0.5μg T_3 + 1.0μg T_4	Group A: N=6 TH-injected embryos nonviable by E17 (100% viable after 1st injection; 50% viable after 2nd injection; 0% viable after 3rd injection) Group B: N=7 TH-injected embryos nonviable by E18 (70% viable after 1st injection; 14% viable after 2nd injection; 0% viable after the 3rd injection)

Table 1 *cont'd*

Trial	Design	Rationale	Outcome
Trail 4	Group A: N=7 saline-injected E12, E14, E16 and E18 Group B: N=7 (0.0625µg T_3 + 0.125µg T_4) E12, E14, E16 and ~~E18~~ Group C: N=7 TH-injected (0.25µg T_4) E12, E14, E16 and ~~E18~~ **did not inject E18 due to low viability*	We had more success with Trial 3 than previous trials, but still no viability by the end point. Since decreasing the dose was more successful, decided to decrease to 1/8th the total dose over 4 injections days to have half the overall dose. Although considered the T_3 to be too potent (since the active form of TH) so wanted to try T_4 injections only (which would mimic the form used to treat hypothyroidism.	Group A: N=7 saline-injected embryos viable at E19 (100%) and tissues collected for controls (perfected techniques and took additional tissues than Trial 1 – including long bones) Group B: N=1 TH-injected embryo viable at E19 (85% viable after 1st injection; 30% viable after 2nd injection; 14% viable after 3rd injection) Group C: N=1 TH-injected embryo viable at E19 (100% viable after 1st injection; 57% viable after 2nd injection; 14% viable after 3rd injection)
Trial 5	Group A: N=7 (1.0µg T_4) E11 only Group B: N=7 (0.5µg T_4) E11 and E15 Group C: N=7 (0.25µg T_4) E11 and E15	While Trial 4 resulted in viability at the end point, it was only 14% which is not ideal for collecting a reliable sample size for analysis. Viability reported so far has been overall between injection days. However, it was also observed the viability declines gradually between injection days. This indicates that the TH is having a prolonged effect. We hypothesize that the additional injections are amassing and becoming toxic over time. Therefore, this trial, continuing to inject T_4 only (most successful), at a low dose, with an extended period between injection days and the end point	*Currently ongoing*

In conclusion, we have been making a great deal of progress and are getting closer to establishing our model for *in utero* thyrotoxicosis in chicken embryos in hopes of collecting samples to compare expression levels of *FGFR1* and *TRα* to our successful saline-injections in support of our model (Trials 1 and 4). It is expected that by December 2020, we should have a model established, and by March 2021, we will have the gene expression data and analysis completed. Although we are not able to complete Aim1 due to the ongoing pandemic, our study evolved into established a model that will support future funding for analyzing skull morphology to determine if in addition to changes in gene expression levels, we can observed morphological differences that would support the proposed mechanisms of TH-induced craniosynostosis with *in utero* thyrotoxicosis. Undergraduate students continue to work on this project and Hannah Ankrom has applied for the NASA WVSGC Undergraduate Affiliate Fellowship. I have also applied for the NASA WVSGC Research Enhancement Award to continue to fund this ongoing project.

Figure 1. Undergraduate student Hannah Ankrom collecting sutural membrane from the chicken embryos at E19 (Trial 1). After removal, membranes were weighed and homogenized. Hannah also was proficient at collecting the blood by cardiac puncture.

Figure 2. Undergraduate student Carson Corrick collecting hearts and livers from the chicken embryos at E19 (Trial 1). After removal, organs were weighed and homogenized.