**Transplant Date: June 29, 2021**

**Protocol Number:** AMI-018-006

**Study Number:** AMI-018-006-117

**Title of Study:** Transplantation with PHH lot DJW using a new blended NTBC cycle tests to evaluate engraftment and early pHH expansion and survival at ≥ 70 days post-transplant.

**Primary Investigators:** Lisa Wilson

**Number of Animals:** 40. **Species:** Rat. **Strain:** SD FRG. **Gender:** Male & Female. **Age or weight range: 5-**6 weeks

**Purpose**:

In IACUC protocol AMI-018-006, the focus of the set of experiments is to utilize the immunodeficient rat model of hereditary tyrosinemia type 1 (HT1) - which are deficient in the enzyme fumarylacetoacetate hydrolase (FAH) – as the model for expanding human hepatocytes. Hepatocytes that are FAH+ have a selective growth advantage over FAH- hepatocyte; therefore, we hypothesize that FAH+ human hepatocytes will grow and repopulate the FAH-KO rat liver. Proof-of-concept has already been demonstrated in the mouse model of HT1 (see Azuma et al. 2007 PMID: 17664939).

Studies are under way to improve the health and survival of transplanted FRG rats out to 125 days post-transplant while maintaining robust expansion of the transplanted primary human hepatocytes. Data from the most recent Phase 1 and 2 NTBC cycle, Cycles 4 and 11: 0.4 > 0.05mg/L (studies 081-101), has high survival and sufficient hAlbumin levels at day 28- and 50-days post-transplant compared to previous NTBC cycles used in studies AMI-018-006-079 and early. To date, we have not been able to improve survival at ≥ 70 days post-transplant. Data from evaluation of blood and tissue at the end of each cycle indicates that injury to kidneys and liver is occurring as early as 28 days post-transplant leading to a progression of chronic health issues due to insufficient NTBC to clear the toxic metabolites and allow for a “rest” period.

Our hypothesis for study AMI-018-006-117, is the same as stated in AMI-018-006-115 and AMI-018-006-116, to provide a higher level of NTBC at the beginning of Phase 3 and 4 to provide the “rest period” that allows the toxic metabolites to clear and reduce the damage to internal organs, especially the liver and kidney. In addition, we need to ensure that the concentration and duration of high NTBC does not reduce the selective pressure on the human hepatocytes to proliferate. We know that a significant drop from high NTBC to low NTBC leads to high morbidity and mortality. This is probably due to a high concentration of NTBC initiating allowing the rat hepatocytes to become metabolically functional then with the immediate switch to 0.05mg/L in conjunction with the short half-life of NTBC, the FRG rat hepatocytes quickly enter an apoptotic state leading to acute liver failure. By applying a blended cycle, 2mg/L > 0.4mg/L > 0.05mg/L, we will allow for the rat hepatocytes to become senescent during the 0.4mg/L NTBC dosing before switching to the 0.05mg/L NTBC to induce selective pressure on the human hepatocytes to proliferate. If our hypothesis is correct the blended cycles during Phases 3 and 4 should improve health and survival at ≥ 70 days post-transplant.

Forty FRG rats will be preconditioned with 1E+8pfu/g of BW and transplanted with a validated commercial lot of pHH. The animals will be cycled using Cycle 11 for the first 52 days. At the start of Phase 3 (71d) and 4 (92d), 0.4mg/L will be replaced with 2mg/L NTBC for 3 days, step down to 0.4mg/L for 4 days and then continue with 0.05mg/L for 14 days.

At the end of each phase, 1 through 4, using a random 5 animals from each group urine will be collected for biomarkers of kidney disease. From the same animals, 500µL of whole blood for serum isolation will be collected via the retro-orbital vein and dispensed into 210µL aliquots. The serum will be submitted for liver comprehensive clinical chemistries and succinylacetone levels and the urine for evaluation of a kidney toxicity panel. This analysis will provide health data that will indicate if future changes at specific phases in the NTBC cycle is necessary to improve health leading to improved survival at ≥ 70 days post-transplant.

Hypothesis AMI-018-006-117: The hypothesis to provide high concentrations of NTBC at the start of Phase 3 and 4 to allow a rest period for the animals before going to the lower NTBC concentrations of 0.4mg/L and 0.05mg/L.

All FRG rats will be on 5LJ5

All FRG rats will begin on 16 mg/L NTBC + 820µg/mL Equisul in the drinking water

**Experiment Outline:**

N=40 FRG rats

Animals will be held for at least one week to acclimate following arrival.

Animals will be socially housed. On the rare case where one animal remains in a cage without a partner, additional cage enrichment (non-food) will be provided.

Animals will be divided into 2 groups, A and B, with equal number of males and females.

Timeline

Description automatically generatedUsing body weights collected the same day as preconditioning the rederived FRG rats from Envigo will be dosed with Ad-uPA 24h ± 2h prior to transplant and placed on the drinking water as specified in the NTBC cycling protocol located on the Ambys G-drive. On the day of transplant, the cryopreserved validated commercial PHH Lot will be prepared following the standard protocol used for transplantation

**Day -1:** In the AM, ALL rats will be dosed with 1E8pfu/gram of body weight Ad-UPA by IV tail injection. Ad-uPA will be provided to the IVS team for tail vein dosing. The NTBC drinking water will be changed as indicated in the above chart.

**Day 0:** Hepatocytes (5 million per 100g of rat) will be prepared in hepatocyte media (2.5 million per 100 µl media). The volume of hepatocytes suspension determine by body weight will be in 1.5ml Eppendorf tubes and transferred on ice. Hepatocytes will remain on ice until time of injection. At time of injection, hepatocytes will be gently pipetted up/down x3 with P1000 pipet and sterile P1000 tip to get the cells in suspension (hepatocytes are large and will quickly pellet to the bottom of the tube). Hepatocytes will be drawn into a sterile 1cc syringe with a 27G needle and injected into the spleen via laparotomy method.

**Day 1 onwards:** Animals will be put on the short NTBC cycle as indicated above in the chart and as indicated in the NTBC water cycle sheet located on the Ambys’ G drive.

All animals post-transplant will be offered the nutritionally balanced Supreme mini treats supplement at the time of body weight collection as positive reinforcement (~1-2 pellets) and for supplementation of additional calories and vitamins at a dose of 6-8 pellets per animal.

The IVS team will record the date and quantity of supplement administration, and clinical observational scores.

**Day 28-92: Urine and Serum collection**

On the days indicated in the chart below 5 animals from Group A and 5 animals from Group B will be randomly selected by the PI or IVS supervisor. Using the standard urine collection method as much urine as possible will be collected from each animal and dispensed into 50µL aliquots before storing at -20C. The animals will be induced to a surgical plane of anesthesia with 3-5% isoflurane and ~500µL of whole blood collected via the retro-orbital vein. Serum will be isolated and 210µL dispensed sterile 1.5mL microfuge tubes before storing at -20C.

|  |  |  |  |
| --- | --- | --- | --- |
| Phase | Day of collection ± 1 days | Group A | Group B |
| 1 | July 28, 2021 | 5 | 5 |
| 2 | August 18, 2021 | 5 | 5 |
| 3 | September 8, 2021 | 5 | 5 |
| 4 | September 29, 2021 | 5 | 5 |

**Day 30 – 90**: 5µL whole blood for human albumin measurements will be collected by tail vein, tail or saphenous vein nick at the specified dates on the chart above and the NTBC water cycle sheet located on the Ambys’ G drive.

**Day 50 huAlb readout:** hAlbumin **≤ 250µg/mL** will be removed from study and euthanized.

**Day 70 huAlb readout:** hAlbumin **≤ 750µg/mL** will be removed from study and euthanized.

**Day 0-125:** Body weights and clinical scoring will be measured and documented up to three times each week.

**Day >125:** IVS to notify PI and Perfusion Team of any remaining animals.

**Animal care and welfare:**

Refer to **the document titled “VETERINARY INSTRUCTIONS FOR CLINICAL CARE AND REPORTING FOR FRG RATS”** for the evaluation and documentation of clinical status and palliative treatments**.** In reference to the group A and B assignments above, animals will be supplemented based on their group assignments.

**Additional Veterinary Testing:**

For veterinary health and surveillance screening, the Attending Veterinarian may request aseptic sample collection of tissues in lieu of perfusion, under general anesthesia. Once all samples have been collected, the animal will be euthanized via exsanguination/vital organ removal.

**Humanized FRG rats for perfusion, hepatocyte isolation and human hepatocyte purification:**

At ≥ 71 days post-transplant with human albumin concentrations of ≥ 2,500µg/mL, animals may be offered to the Product Development team for optimization of processes involving liver perfusion (refer to the document titled “Perfusion Procedure Guideline: Portal Vein and Inferior Vena Cava Approaches), hepatocyte isolation, human hepatocyte purification and in vitro characterization.

Once the cannula has been inserted into the caudal vena cava or portal vein, secured and blood starts to fill the head, ≥ 500µL whole blood will be collected for sera isolation. Alternatively, the blood may be collected from the tail vein based on a 50µL aliquot of sera will be dispensed into a pre-labeled sterile 1.5 mL tube for Tyrosine and Succinylacetone quantitation. Remaining sera will be transferred to a second pre-labeled sterile 1.5mL tube. Both aliquots should be stored at -20C until shipped for analysis.

Principal Investigator: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Head of Pharmacology (or alternate): \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_

**Appendix A: Processing blood, urine and tissue samples**

Blood collection

* Using Isoflurane, anesthetize the animal to a surgical plane of anesthesia (Induction 4-5%, maintenance 2-3%). Confirm with a firm toe pinch on both hind feet.
* Using operating scissors or other appropriately sized scissors, cut through the skin and muscle to open the peritoneal cavity.
* Using gauze, sweep the intestines out of the body cavity to expose the inferior vena cava.
* Using 5 mL (rat) syringe and 25 G needle puncture the inferior vena cava and slowly withdraw 4mL of blood from rat. Be careful to not collapse the vein by creating too much vacuum pressure.

Serum:

* Dispense ≤ 2mL whole blood volume into 4mL serum separator tube and allow to clot for at least 1 hour at room temperature.
* Centrifuge at 10,000 rpm for 5 minutes.
* Dispense 220µL into 4 X 1.5 sterile mL tube, store at -80C

Plasma:

* Dispense ≥ 1mL of whole blood in a 4mLK2EDTA anticoagulation micro tube (do not overfill or blood will clot).
* Gently invert 3 times (do not shake) to facilitate complete mixing with anticoagulant.
* Centrifuge at 10,000 rpm for 5 minutes.
* Dispense 80µL into 4 X 1.5 sterile mL tube, store at -80C

Urine during Necropsy

* Following collection of blood, collect urine (if present) via cystocentesis. Urine to be placed in labeled freezer compatible tube and store at -20oC

Collection of Liver

* Using blunt tipped scissors (curved if possible) separate the liver from the diaphragm, blood vessels and connective tissue and remove the liver from the body cavity.
* Make 4-5, 1cm nicks across the liver to facilitate fixation.
* Place whole liver in bottle of 10% NBF.

Collection of the Kidney

* Remove the adrenal glands, located in the fat cranial to the kidneys.
* Dissect the kidneys away from the fat and cut at the juncture of the kidney with the renal artery and vein.
* Transect the left kidney (transverse section) with a scalpel blade or straight razor.
* Longitudinally bisect the right kidney at the midline.
* Place into a bottle with 10% NBF