Pivotal Preclinical Trial of the Spheroid Reservoir Bioartificial Liver

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Supplemental Material 1: Impact of Dietary Protein on Ammonia Detoxification

Methods

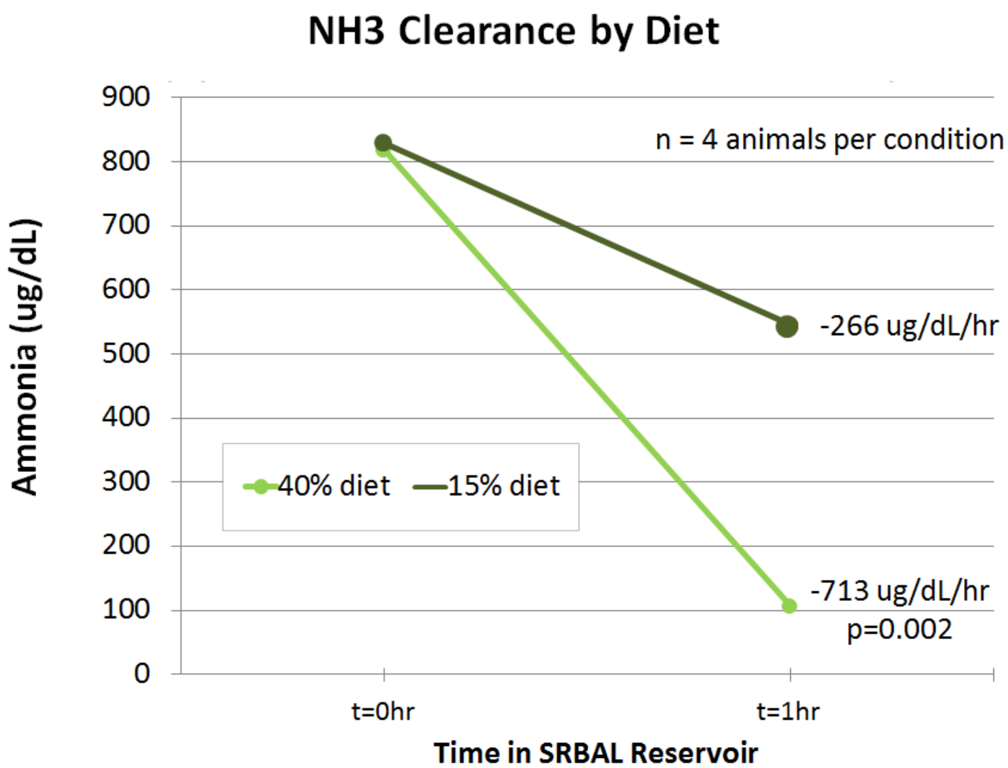
Prior to initiation of the RALF study, eight hepatocyte harvests were conducted to compare the impact of dietary protein on ammonia detoxification of harvested hepatocytes. Donor animals were fed diets of either 15% protein (n=4) or 40% protein (n=4) for seven days prior to hepatocyte isolation. Hepatocytes procured from these animals were rocked to form spheroids and media samples were obtained for bench testing. Ammonia concentrations in media were quantified by AMON slide assay (Product #1721869, Vitro Chemistry Products) on the Vitros 5600 Integrated System (www.orthoclinical.com). Methods of hepatocyte isolation and spheroid formation are described in the main text of the paper.

Results

As illustrated in Fig. S1.1, spheroids procured from pigs fed a 40% protein diet had greater magnitude of ammonia detoxification (-713 ± 122 µg/dL/hr) compared to spheroids from pigs fed a 15% protein diet (-266 ± 30µg/dL/hr, p=0.002). A decline in average ammonia concentration from 800 µg/dL to 100 µg/dL was observed after 1 hour of culture. Donor animals used for the SRBAL study received the high protein diet following this observation.

Fig. S1.1 Ammonia Clearance is Increased by Dietary Protein in Pigs.

Rate of ammonia detoxification of hepatocyte spheroids used in SRBAL was increased significantly by increasing the dietary protein content of donor pigs from 15% to 40%. Experiments were performed in quadruplicate using rocked suspension cultures of hepatocyte spheroids at 5x10^6 cells/mL x 1000mL of medium supplemented with ammonia (800 µg/dL).



Supplemental Material 2: Biochemical Levels during Extracorporeal Therapy

Methods

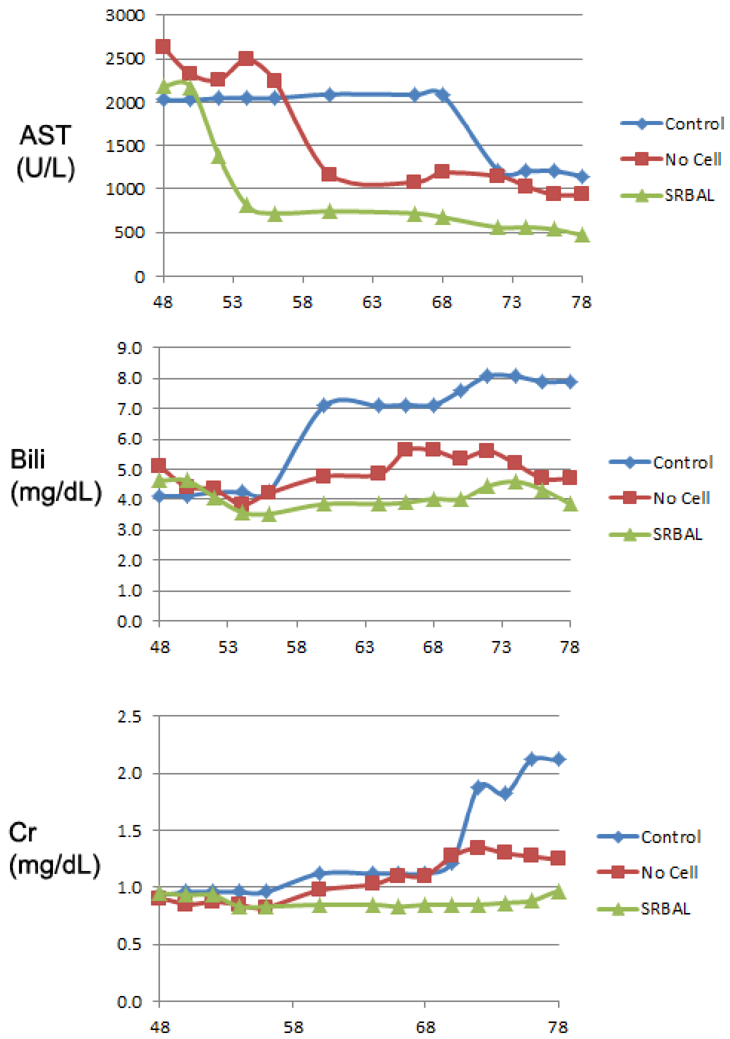
Animals were monitored every two hours during extracorporeal therapy initiated at 48 hours after administration of D-galactosamine. Monitoring steps included blood draws. Chemical analysis (AST, total bilirubin, Cr) was performed in the Mayo Clinical Chemistry Lab.

Results

Fluctuations in mean levels of AST, total bilirubin, and creatinine after initiation of therapy are shown in Fig. S2.1. No significant differences in these three parameters were observed between groups from 48 to 78 hours.

Fig. S2.1 Mean Levels of AST, Total Bilirubin, and Creatinine.

Mean levels of AST, total bilirubin (Bili) and creatinine (Cr) during the treatment interval are shown as line diagrams. Levels of all three biochemical markers were similar at onset of therapy (t=48hr). N=6 animals per group.



Supplemental Material 3: Plasma Ammonia – Option A vs Option B

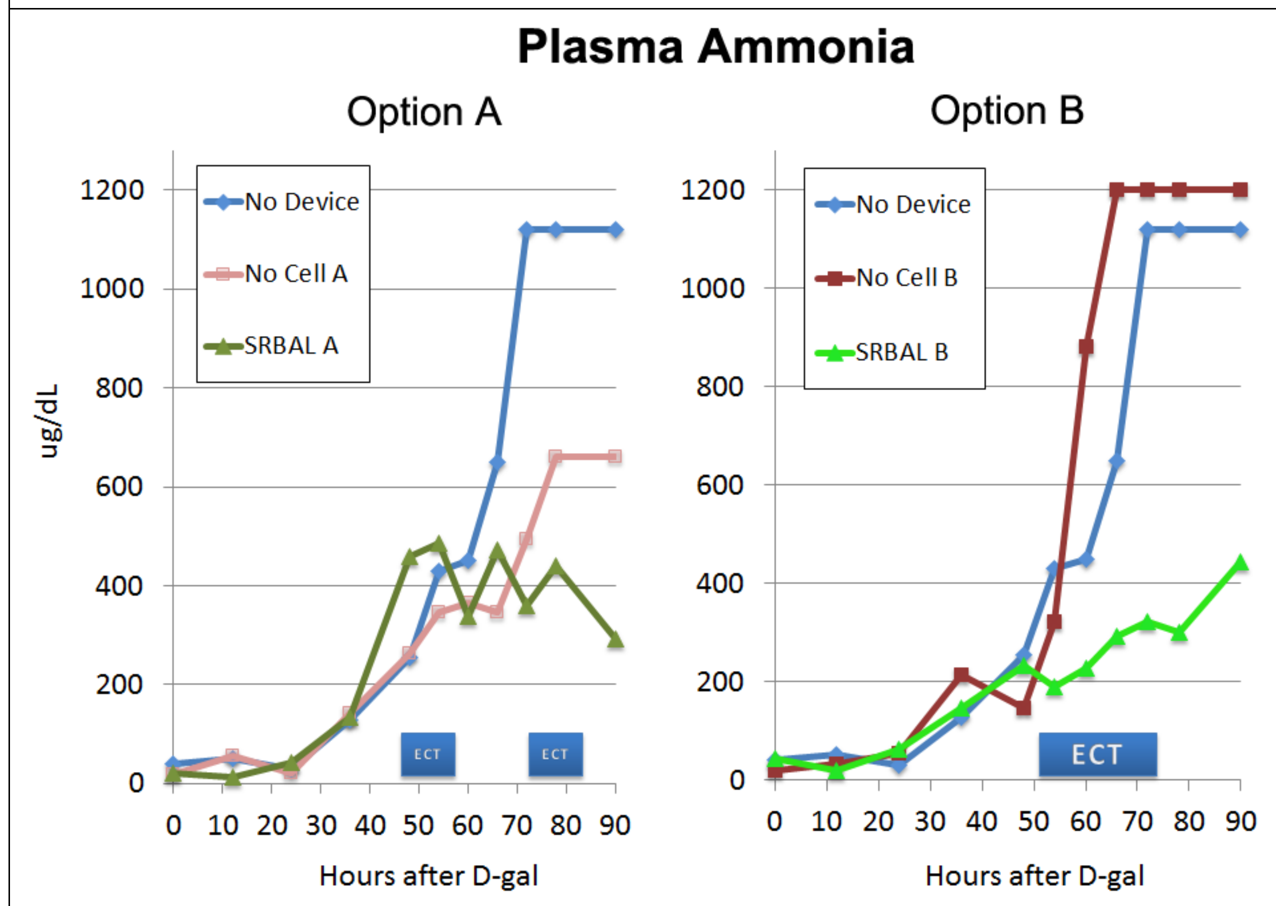
Methods

Animals were monitored every 6 hours for the first 24 hours after administration of D-galactosamine, and then every two hours including extracorporeal therapy initiated at 48 hours. Monitoring steps included blood draws for ammonia performed in the Mayo Clinical Chemistry Lab. Ammonia concentrations in plasma were quantified by AMON slide assay (Product #1721869, Vitro Chemistry Products) on the Vitros 5600 Integrated System (www.orthoclinical.com). Last observation carry forward (LOCF) was used in case of missing values. Missing values were anticipated before starting the study in case of deaths before 90 hours.

Results

Animals in SRBAL treatment Options A and Option B groups both received a significant detoxification benefit of therapy as their plasma ammonia levels remained lower than corresponding control groups throughout the treatment interval (Fig. S3.1).

Fig. S3.1 Plasma ammonia. Median values of plasma ammonia were lower in SRBAL devices compared to No cell devices and no device control animals under both Option A and Option B treatment conditions. Scheduled treatment intervals are shown in blue blocks. Data points are the median value of n=3 measurements per group.



Supplemental Material 4: Brain Water Content and ICP

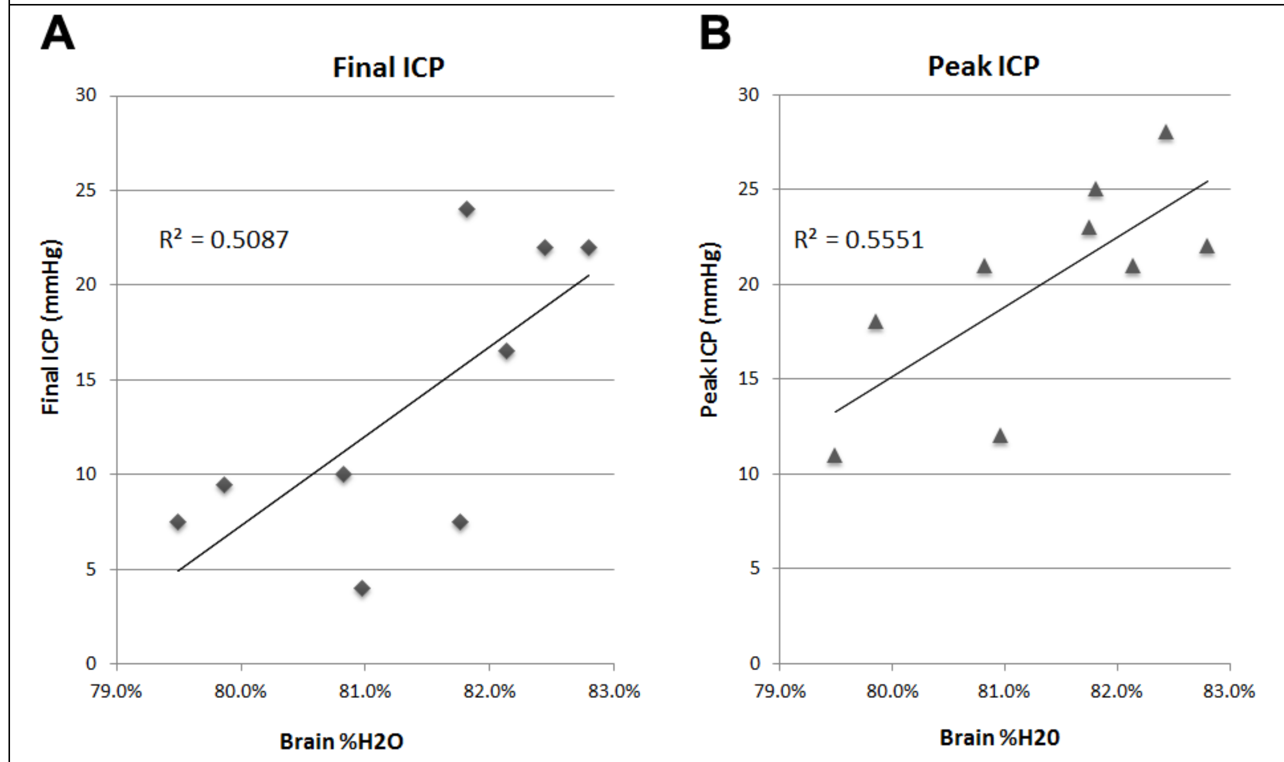
Methods

All animals underwent general anesthesia (inhaled isoflurane 2-3%, induction: 5mg/kg Telazol, 2 mg/kg Xylazine, 0.01 mg/kg Glycopyrrolate) for the placement of an ambulatory intracranial pressure (ICP) monitoring device (RAUMEDIC, Germany) and an internal jugular central venous access r (Palindrome™ 55cm, Covidien, Mansfield, MA, www.kendallhq.com) one week prior to induction of ALF. Animals were monitored every 6 hours for the first 24 hours after administration of D-galactosamine and every two hours for the remainder of the study. Propofol (0.1-0.2 mg/kg/min) was administered briefly via the central venous line to assure lateral decubitus positioning for all ICP measurements. Reported values of ICP (mmHg) were the average of 3-5 measurements obtained over a 2 minute interval. Brain %H_20 was determined on brain white matter by gravimetric technique immediately after an animal met its study endpoint. Correlation coefficients (R^2) were determined from linear regression fit of brain %H_20 and ICP data.

Results

The correlation coefficients (R^2) of brain %H_20 to final ICP and brain %H_20 to peak ICP were 0.51 (p=0.16) and 0.56 (p=0.13), respectively, as shown in Fig. S4.1.

Fig. S4.1 Correlation Between Brain water Content and ICP. Panel A - Direct correlation was observed between brain %H_20 and final ICP. Panel B - Direct correlation was also observed between brain %H_20 and peak ICP. Missing data points reflect animals from which fresh brain sampling was not possible.



Supplemental Material 5: ROS Levels during Treatment Interval

Methods

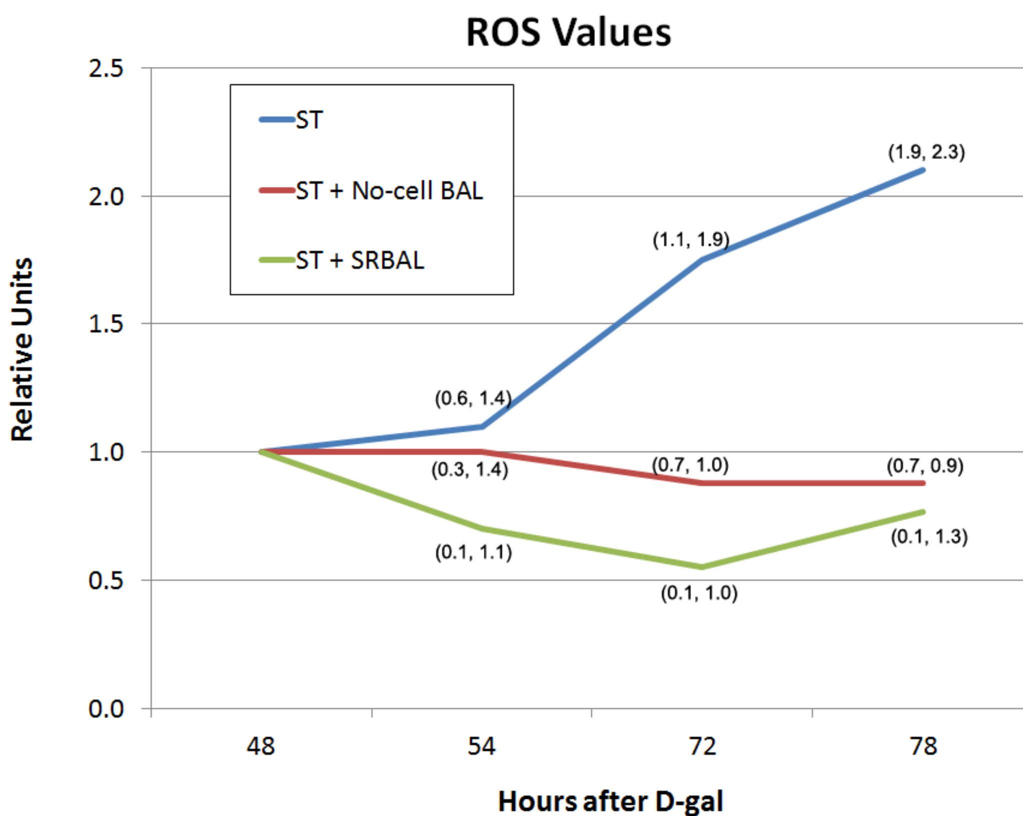
Animals were monitored for ROS levels during extracorporeal therapy. ROS were quantified by an established microplate technique involving dichlorofluorescein diacetate (DCFDA; Invitrogen, Carlsbad, CA) and dichlorofluorescein (DCF) (1). Substrate material (100 μM DCFDA) was incubated 30 min in the dark with hydroxylamine hydrochloride (40 mM) and hydrolyzed to non-fluorescent DCF. DCF was incubated with plasma samples and oxidation rate was recorded by changes in fluorescence over a 10 min period with a spectrofluorometer (BioTek, Winooski, VT) at 485 nm excitation and 520 nm emission wavelengths.

Results

ROS levels measured during the treatment intervals are summarized in Fig. S5.1. Pigs treated with an albumin-based extracorporeal device (SRBAL and the No-cell device) had significantly lower blood levels of ROS at the completion of therapy compared to ST alone ($p<0.05$).

Fig. S5.1 ROS during Treatment Interval.

Treatments were initiated at 48 hours and completed by 78 hours. Median ROS values (min, max) of each treatment group are shown. N= 6 data points per group. These patterns suggest a device effect on ROS levels.



References

1. Wang H, Joseph J. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. RFree Radic Biol Med 1999;27:612-616.

Supplemental Material 6: Cytokine Levels during Treatment Interval

Methods

Pro-inflammatory cytokines levels were monitored in ALF animals during extracorporeal therapy. Levels of four porcine proinflammatory cytokines (IL1β, IL6, IL18, TNFα) were quantified by enzyme-linked immunosorbent assay kits (product #'s ELP-IL1b, ELP-IL6, ELP-IL18, ELP-TNFa, RayBiotech, Norcross GA).

Results

Average levels of pro-inflammatory cytokines (IL-1B, panel A; IL-6, panel B, IL-18, panel C) are summarized in Fig. S6.1. Levels of these three cytokines rose with onset of ALF after infusion of D-gal at t=0hr. Levels of these three cytokines were not impacted by treatment condition. Levels of TNFα were low in ALF pigs and generally below the detection limits of the assay (not shown).

Fig. S6.1 Levels of IL1β, IL6, IL18 during Treatment Interval.

Panel A – Plasma levels of IL-1B.

Panel B – Plasma levels of IL-6.

Panel C – Plasma levels of IL-18.

Data points are mean values of 6 measurements per group. Error bars are standard deviation of the mean.