Title: Development of a wearable bioartificial kidney using the Bioartificial Renal Epithelial Cell System (BRECS)

Short Title (70 characters): A wearable kidney using a Bioartificial Renal Epithelial Cell System

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Cell therapy for treatment of renal failure in the acute setting has proved successful with therapeutic impact, yet development of a sustainable, portable bioartificial kidney for treatment of chronic renal failure has yet to be realized. Challenges in maintaining an anticoagulated blood circuit, the typical platform for solute clearance and support of the biological components, have posed a major hurdle in advancement of this technology. Our group has developed a Bioartificial Renal Epithelial Cell System (BRECS) capable of differentiated renal cell function while sustained by body fluids other than blood. To evaluate this device for potential use in end stage renal disease, we established a large animal model that exploits peritoneal dialysis fluid for support of the biologic device and delivery of cell therapy while providing uremic control. Anephric sheep received a continuous flow peritoneal dialysis (CFPD) circuit that included a BRECS. Sheep were treated with BRECS containing $1x10^8$ renal epithelial cells or acellular sham devices for up to 7 days. BRECS cell viability and activity were maintained with extracorporeal peritoneal fluid circulation. A systemic immunologic effect of BRECS therapy was observed as cell treated sheep retained neutrophil oxidative activity better than sham treated animals. This model demonstrates that use of the BRECS within a CFPD circuit embodies a feasible approach to a sustainable and effective wearable bioartificial kidney**.**

1. INTRODUCTION

Renal replacement therapy (RRT) using hemodialysis or peritoneal dialysis (PD) has become a mainstay therapy for patients with End Stage Renal Disease (ESRD). Although this approach is life sustaining, it remains suboptimal with poor clinical outcomes. Current RRT utilize semipermeable membranes to substitute for the small solute clearance function of the renal glomerulus but they do not replace the transport, metabolic, and endocrinologic functions of the tubular cells, rendering them only partial substitutive therapy at best. The better outcomes in patients treated with kidney transplant compared to chronic dialysis emphasizes the benefits of full renal replacement for ESRD (US Renal Data System 2008; Tonelli et al. 2011).

Renal cell therapy incorporated into conventional RRT has shown metabolic, immunologic and survival benefits in acute renal failure (ARF) in preclinical (Humes et al. 1999; Humes et al. 2002; Humes et al. 2003a; Fissell et al. 2003; Huijuan et al. 2007; Wang et al. 2010; Westover et al. 2014) and clinical studies (Humes et al. 2003b; Humes et al. 2004; Tumlin et al. 2008). The first renal cell therapy device used in patients with ARF, the Renal Assist Device (RAD), demonstrated that allogeneic renal epithelial cells (REC) maintained within an extracorporeal environment could provide therapeutic benefit to patients requiring continuous RRT and improve survival (Humes et al. 2004; Tumlin et al. 2008). The limitations of cell

expansion and device design made manufacture, storage, and distribution of the RAD a challenge to meet clinical demand. Enhanced REC propagation methodologies (Westover et al. 2012), along with improved fabrication techniques have overcome these challenges giving rise to a second generation device, the Bioartificial Renal Epithelial Cell System (BRECS), which was designed to surmount a number of obstacles preventing widespread use of cell based therapies. This approach resulted in the ability to maintain a dense population of REC within a compact, portable and cryopreservable format for on-demand deployment in clinical situations (Buffington et al. 2012). The BRECS is a bioreactor containing adult REC seeded onto porous carbon disks within a polycarbonate housing. Cell viability is maintained by perfusion culture through the disks, allowing cells to respond to alterations in the perfusate milieu, potentially releasing metabolic and endocrinologic products with therapeutic value.

In addition to previously mentioned limitations for global use of cell enhanced therapies, the difficulty in maintaining continuous extracorporeal blood circulation without thrombosis, despite continuous anticoagulation strategies, remains an impediment to development of a sustainable portable cell device for treatment of ESRD. Elimination of the blood circuit inherent to hemodialysis based therapies could facilitate delivery. To this end, a wearable bioartificial kidney (WeBAK) that exploits peritoneal fluid to maintain oxygen and nutrient delivery to a BRECS was

conceived and tested in an ovine model. BRECS devices were monitored during and after extracorporeal culture and serial blood sampling was performed on sheep to assess metabolic, endocrinologic and immunologic impact of BRECS therapy. The project aimed to provide insight into the feasibility and potential impact of delivering renal cell therapy in this manner for treatment in ESRD.

2. MATERIALS AND METHODS

2.1 Bioartificial renal epithelial cell system (BRECS)

The BRECS is comprised of porous carbon disks colonized with up to 2 x10^8 human REC within a perfusable polycarbonate housing. Perfusate flows over and through the disks to sustain cell viability and export cell products. An injection molded design allows BRECS to be fitted with monitoring systems, permitting monitoring of temperature and oxygen consumption in real time. Total weight of a cell seeded and fluid filled device is approximately 62gm.

Primary REC were isolated and expanded from human kidneys unsuitable for transplantation due to anatomic or fibrotic defects (procured from the National Disease Research Interchange) following an established method (Westover et al. 2012). Details of REC isolation, seeding onto carbon disks for use in BRECS units and maintenance of BRECS *in vitro* have been described (Buffington et al. 2012).

Metabolic activity of REC within BRECS was evaluated using oxygen consumption rates (OCR) and non-destructive glutathione (GSH) metabolism. Oxygen measurements were performed either using an i-STAT analyzer (Abbott Point of Care, Princeton, NJ) on media collected from closed units or by oxygen sensors. RedEye patches (Ocean optics, Dunedin, FL) or PSt3 patches (PreSens, Regensburg, Germany) were set up in recirculating, closed, oxygen impermeable circuits. To determine average OCR, a linear regression approximation of slope was used. GSH metabolism was determined by the rate of degradation of exogenously added GSH. BRECS were completely filled with 20µM GSH (Sigma-Aldrich) in Ultra MDCK media and samples collected at baseline, 10 and 30 minutes. Samples were analyzed by the method of Tietze (Tietze 1969). After extracorporeal culture, a qualitative assessment of cell viability was made by adding 1 µg/mL fluorescein diacetate and propidium iodide to individual disks from disassembled BRECS within well plates. Living and dead cells were visualized immediately with a Zeiss Axiovert 200 inverted fluorescence microscope (Carl Zeiss, Inc. Thornwood, NY) equipped with corresponding filter sets, and micrographs obtained using Zeiss AxioCam MRm and ICc1 cameras (Carl Zeiss, Inc.).

2.4 Experimental animals and the extracorporeal WeBAK circuit

Animal use adhered to principles stated in the Guide for Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, 1996) and procedures were performed under protocols approved by the institutional committee for care and use of animals at the University of Michigan. Female sheep (35-45 kg) were maintained under standard laboratory conditions until onset of continuous flow peritoneal dialysis (CFPD), during which they were confined in customized stanchions while connected to the extracorporeal circuit. Sheep were instrumented with separate ingress and egress PD catheters and nephrectomy was staged to permit healing around the catheters which were placed concurrent with removal of the first kidney. Antimicrobial and analgesic medication was administered peri-operatively. CFPD was instituted within 24h of complete nephrectomy using commercially available 4.25% glucose dialysate solution (Dianeal® Baxter Healthcare, Deerfield, IL) with nafcillin [100mg/L] and gentamicin [4mg/L] added. An intraperitoneal instillation of 2-3L dialysate was continuously recirculated at 80-100ml/min through a primed extracorporeal circuit of ¾" Tygon® tubing using a rotary pump with a segment of collapsible tubing to prevent generation of negative abdominal pressure (M-pump, MC3 Inc., Ann Arbor, MI). A polysulfone hemofilter with a 65kD molecular weight cut-off provided immunoisolation for the cells by generating ultra-filtered PD that was directed by a peristaltic single channel pump (Masterflex® Cole-Palmer, Vernon Hills IL) through

a fluid warming system (Hotline®, Smith Medical) at 38^0C for parallel perfusion of the BRECS (Figure 1). Total circuit volume was approximately 1000mL. Dialysate was refreshed by continuous flow into and out of the circuit plus daily exchanges of the abdominal content. BRECS containing approximately $1x10^8$ REC or acellular sham devices were cultured under identical conditions *in vitro* prior to placement in an extracorporeal WeBAK circuit. Devices were deployed subsequent to dialysate equilibration to physiologic pH and were maintained by ex vivo perfusion with peritoneal fluid for up to 7 days.

Serum chemistry and electrolyte values were determined using automated veterinary analyzers (IDEXX VetTest®, IDEXX VetLyte®, IDEXX Laboratories Inc. USA) according to the manufacturer's directions. Complete blood counts and differentials were determined by a Hemavet® analyzer (Drew Scientific, Waterbury CT). Neutrophil (NE) counts and quantification of intracellular reactive oxygen species (ROS) were assessed as markers of immunologic status. NE were isolated on a discontinuous Percoll gradient, incubated with the reactive dye, 5-(and 6)-chloromethyl-2', 7'-dichlorodihydroflourescein diacetate, acetyl ester (CM-H2D CFDA, Molecular Probes) [10uM] in RPMI 15min at 37°C, followed by incubation (+/-) phorbol-12-myristate-13-acetate (PMA) [1uM] at 37°C for 30min. NE were labeled with mouse-anti-bovine unconjugated CD11b (ABD Serotech) /Anti-IgG

conjugate mouse 647 (Life Technologies) to allow gating then fixed. Mean fluorescent intensity was determined using an Accuri Flow Cytometer.

Endocrine support by BRECS was assessed using Vitamin D3. To ensure an adequate source of substrate, 3000 units/day of 25-hydroxyvitamin D3 (25VitD3, Sigma Aldrich) was infused into the CFPD circuit pre BRECS. Sheep serum samples were frozen and sent to Heartland Assays, Inc. (Ames, IO) for quantification of 1α,25-dihydroxyvitamin D3 (1,25VitD3) by radioimmunoassay.

2.9 Statistical analyses

Comparisons between groups were made using Student's t test assuming equal variance. Significance was set at $p<0.05$.

3. Results and Discussion

3.1 Establishing a WeBAK using CFPD in an ovine model

Tissue engineered devices, such as the RAD and the BRECS, have revealed the therapeutic value in replacing the lost metabolic, endocrine and immunologic functions of the kidney, however impediments to long term delivery of cell therapy remain. As technological advancements are made in miniaturization of medical devices, interest in portable dialysis systems is increasing as is recognition of PD as a viable platform for a sustainable wearable artificial kidney (Kim and Ronco

2011; Davenport 2012; Armignacco et al. 2015). A large animal model of uremia sustained using 24h continuously recirculating CFPD was developed to demonstrate a WeBAK based on this modality. Pilot animals (n=13) were needed to establish instrumentation and PD protocols. Catheter complications were a primary obstacle to a recirculating CFPD regimen, with catheter dysfunction occurring in 23 of the 34 total animals used. Leakage of fluid around catheters and catheter occlusion by omental wrapping were leading causes for disruption or failure of CFPD. Inclusion of a purse string suture in the peritoneum at catheter implantation, a 14 day healing period prior to onset of PD, and Oreopoulos-Zellerman catheters that limit omental wrapping were found to collectively minimize catheter malfunction in this model. Fibrin accumulation within the circuit was universal and the filter was replaced as needed to maintain circuit patency. Culture of bacteria from PD fluid of 5 pilot animals prompted empiric addition of antimicrobials to the dialysate. Bacterial culture of PD fluid was positive for a single sheep in each of the study groups, however no microbial contamination of BRECS units was identified, demonstrating efficacy of the WeBAK design to maintain an aseptic environment for the BRECS.

Upon surmounting the challenges of the model, a stable uremic state could be established with CFPD, enabling at least 9 days of study with anephric sheep (Figure 2). Dialysate flow rates into the circuit and to waste were set at 420 and

470mL/hr respectively, providing a calculated weekly creatinine clearance of 55L/wk and ultrafiltrate generation up to 1L/day. As study aims were to determine feasibility and effects of maintaining the biologic device within the WeBAK, further characterization or optimization of dialysis efficiency using the CFPD circuit was not attempted. Uremic control was dependent on dialysis dose and therefore not different between animals treated with cellular or sham devices.

3.3 Extracorporeal maintenance of BRECS

BRECS containing approximately $1x10^8$ REC or acellular sham devices were cultured under identical conditions for an average of 21±9 days *in vitro* according to BRECS protocol prior to placement in an extracorporeal WeBAK circuit. Devices were maintained in the WeBAK by perfusion with ultra-filtered peritoneal fluid for up to 7 days then returned to *in vitro* culture for 48h prior to dismantling for histology. Cell BRECS demonstrated relatively consistent metabolic activity throughout the duration of extracorporeal therapy confirming that REC within the BRECS remained viable and metabolically active when maintained by the nutrients provided by CFPD. At the commencement of extracorporeal culture, OCR in BRECS averaged 129 nmol/min, and by day 7, average OCR was 85 nmol/min (Figure3A). OCR measured on days 5-7 trended toward being slightly lower than initial measurements; however, these values were not significantly lower (p=0.65,

0.53 and 0.59 for days 5-7 respectively). Non-destructive GSH degradation by γ-glutamyltranspeptidase (γGT) was chosen as a representative measurement of BRECS metabolic function. γGT, expressed on the apical brush border membrane of renal proximal tubules, catalyzes the salvage of GSH. GSH depletion in patients receiving maintenance dialysis contributes to the oxidative stress of ESRD patients. Average GSH degradation in BRECS at day 0 was 1194 nmol/hr and 898 nmol/hr on day 7 (Figure 3B). Both OCR and GSH degradation increased toward pre study values when BRECS were removed from sheep and returned to *in vitro* culture. No metabolic activity was detected in sham devices. Histologic staining of individual disks removed from cell BRECS at the conclusion of the therapy period exhibited high densities of living cells following up to 7 days of extracorporeal culture (Figure 4), with few dead cells.

3.4 Impact of BRECS therapy

The BRECS was designed to be the first all-in-one culture vessel, cryostorage device and cell therapy delivery system for clinical perfusion with either ultra-filtered blood or body fluids. BRECS, maintained in a hematogenous circuit has demonstrated therapeutic impact in a preclinical model AKI (Westover et al. 2014) yet this delivery method has limitations, particularly for chronic indications. With the ability of cells within BRECS to be sustained by *ex vivo* peritoneal fluid, we

investigated the potential therapeutic impact of cell enhanced therapy delivered in this manner for ESRD.

Anephric sheep either received cell BRECS (n=13) or sham device (n=8) therapy via the CFPD circuit starting day 1 post nephrectomy. Therapy was continuous for up to 7 days, then devices were removed and sheep sustained with CFPD alone for an additional 48h post therapy. Blood was sampled prior to removal of each kidney then at a minimum of every 48h to assess endocrinologic, metabolic and immunologic parameters. Inability to maintain adequate flow in the CFPD circuit to perfuse devices for a full 7 days caused some animals to be terminated before all sampling was competed therefore data was averaged over each of 3 phases of the study: non-uremic, therapy, and post therapy. Nine cell BRECS and 5 sham treated sheep completed the entire study time course.

VIT D3 levels were measured as an indicator of the endocrinologic support that could potentially be provided by the BRECS. Western blotting of isolated REC has confirmed the presence of 1- alpha hydroxylase, the enzyme that controls the final conversion of 25VIT D3 to the biologically active 1,25VIT D3. Systemic 1,25VIT D3 levels decreased in all sheep post nephrectomy and were highly variable in uremic sheep over the BRECS therapy period with no difference detected between cohorts (data not shown). This may be attributable to seasonal (sunlight) variation which impacts Vitamin D kinetics and the individual animal response to exogenous

precursor supplementation with 25VIT D, which can increase extra-renal 1-alpha hydroxylase activity in whole animal systems (Richart et al. 2007). Of note, activity of 1-alpha hydroxylase was not detectable in BRECS under normal tissue culture conditions however, when phosphate was omitted from the culture media, 1-alpha hydroxylase activity was upregulated inside 24h (unpublished data). Further investigation is needed to evaluate factors that may influence delivery of Vitamin D by BRECS (i.e. phosphate levels in PD fluid).

Circulating NE counts were stable in cell BRECS treated sheep (2.88±0.35 non-uremic, 4.20±1.44 K/uL post-therapy) while NE counts in sham treated animals were increased significantly at post therapy compared to the non-uremic state (6.26±2.0 and 3.01±0.69 K/uL respectively, p=0.017). The differences between cohorts were not significant at any point. Since uremia has been associated with NE dysfunction (Haag-Weber and Horl 1996; Anding et al. 2003), the oxidative activity of isolated systemic NE was assessed by quantification of intracellular ROS. Results are summarized in Figure 5 with values reported as mean fluorescent intensity ± SE both with and without PMA stimulation. ROS data were not available from 4 of the cell treated sheep. The pre therapy values (basal and stimulated) were not different between groups. Basal ROS level was maintained and the ROS production of stimulated NE was significantly greater in sheep receiving BRECS cell treatment both during (Cell 198 +/- 69, Sham 64 +/- 20,

p=0.033) and post therapy (Cell 232 +/- 64, Sham 37+/- 14, p=0.003). Exclusion of immunologic data from the 2 sheep with septic peritonitis did not significantly alter these findings.

4. Conclusions

The BRECS offers the possibility of full renal replacement therapy by providing the transport, endocrine, metabolic and immunologic activity that is lacking in conventional dialysis modalities. BRECS therapy has demonstrated therapeutic benefit in a preclinical model of AKI and BRECS design overcomes a number of obstacles that have deterred clinical administration of renal cell therapy. A key feature of BRECS is the ability for maintaining the cells using body fluids other than blood. Renewed interest in utilization of PD as the basis for an artificial kidney with increased longevity and portability for chronic indications lends a platform for employment of the BRECS to provide cell therapy for patients with ESRD. In this study, we showed that a CFPD regimen was able to manage the uremic state of anephric sheep plus sustain cellular viability and functionality within BRECS as evidenced by oxygen consumption and glutathione metabolism during therapy. Viability stains post-therapy confirmed REC survival when maintained in this manner for up to 7 days. These experiments demonstrate proof of concept of a

WeBAK, free from the constraints of an anticoagulated blood circuit, to provide cellular enhanced therapy for chronic renal failure indications.

Fluid dynamics of the BRECS have been optimized so that perfusion rates as low as 10mL/min are able to provide adequate nutrient and oxygen delivery to cells for survival without the use of an oxygenator. Lower metabolic output by REC within BRECS, though not statistically significant, was observed over the one week course of WeBAK therapy in this model. Correlation of GSH metabolism in BRECS and cell number via DNA isolation (reported previously) suggests the reduction of metabolic output likely was due to a combination of viable cell loss along with lower metabolism by the remaining cells when maintained with peritoneal dialysis perfusate. A decrease in oxygen consumption is also likely a combination of fewer cells consuming oxygen at a lower rate and there may be some shift from aerobic to anaerobic metabolism. Further investigation is needed to determine if metabolic output from BRECS during extracorporeal culture is predictably altered and to determine if the changes in metabolic output have therapeutic implications. Of note, we have since found that including a segment of oxygen permeable silicone tubing pre-BRECS can increase the oxygen tension in the perfusate to approximate room air however studies are need to determine if this modification has any effect on metabolic output from BRECS during ex vivo perfusion with PD fluid.

While the importance of renal tubule cells in glutathione metabolism and activation of Vitamin D3 and are well described, these cells also possess a less recognized, but important role in immunoregulatory function. Demonstrated immunologic effects of renal cell therapy in the setting of AKI have included differences in plasma cytokine concentrations, alterations in release of cytokines from isolated leukocytes and improved survival of sepsis (Humes et al. 1999; Humes et al. 2002; Fissell et al. 2003; Humes et al. 2003; Humes et al. 2003; Humes et al. 2004; Huijuan et al. 2007; Westover et al. 2014). Concomitant immune activation, systemic inflammation and immune deficiency have been linked with ESRD (Kato et al. 2008; Vaziri et al. 2012; Betjes 2013). Uremia appears to impair the phagocytic and killing functions of circulating granulocytes (Porter et al. 1997; Anding et al. 2003; Sardenberg et al. 2006; Vaziri et al. 2012) although conflicting results about the generation of ROS by NE isolated from ESRD patients, particularly among those receiving different dialysis modalities, have been described (Porter et al. 1997; Anding et al. 2003; Morena et al. 2005; Sardenberg et al. 2006; Yoon et al. 2007). Infection is the second most common cause of death in ESRD, approaching 25% of annual mortality rate in hemodialysis patients (U.S. Renal Data System 2003). This infection complication rate is not diminished with higher dialysis dose or high flux membrane utilization. In this project, it was found that basal ROS production of isolated NE declined in sheep over the uremic time

course but was better maintained in the animals receiving cell therapy. Moreover, NE counts remained stable and the stimulated oxidative potential of NE was retained during and after BRECS treatment signifying that the ability to have an appropriate immune response to stimuli may be preserved with REC therapy.

The results of this preliminary study demonstrate that a 24h CFPD regimen is able to provide a stable uremic state in nephrectomized sheep. A BRECS is readily incorporated into an extracorporeal CFPD circuit using peritoneal fluid for perfusion of the device, providing cell survival and maintenance of function while eliminating the requirement for an anticoagulated blood circuit. Cell therapy using BRECS contributes to improved immunologic homeostasis during the uremic state, and endocrine support in the form of 1,25 Vitamin D3 may become an added benefit. Advancement of CFPD (ideally with a dialysate regeneration system based upon sorbent technology) that couples clearance of uremic toxins with perfusion to a cell therapy device, such as the BRECS, embodies a feasible approach to a wearable bioartificial kidney for treatment of ESRD.

DISCLOSURES

H. David Humes is a shareholder of Innovative BioTherapies, Inc. K. Johnston, D. Buffington, A Westover and C. Pino are employees of Innovative BioTherapies, Inc.

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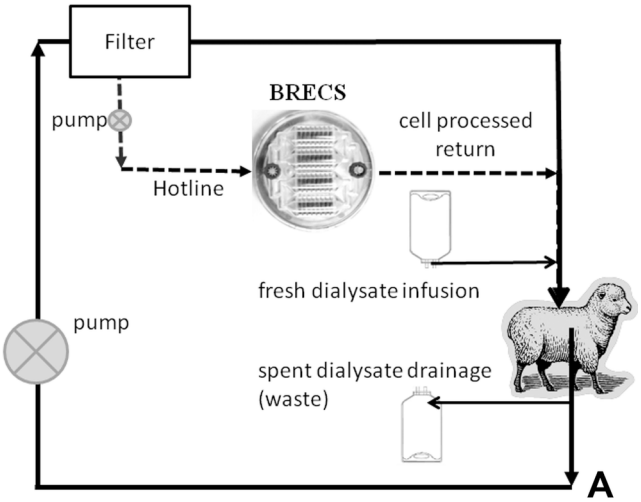

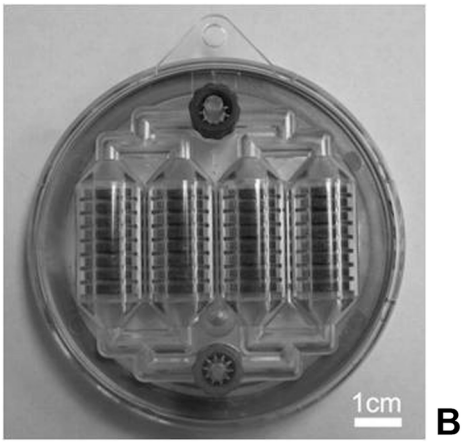

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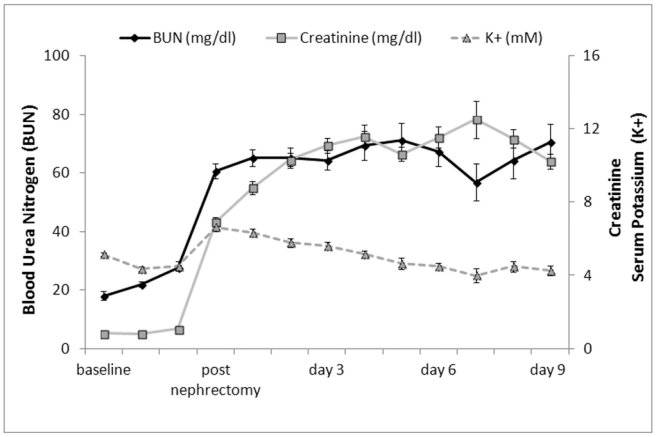

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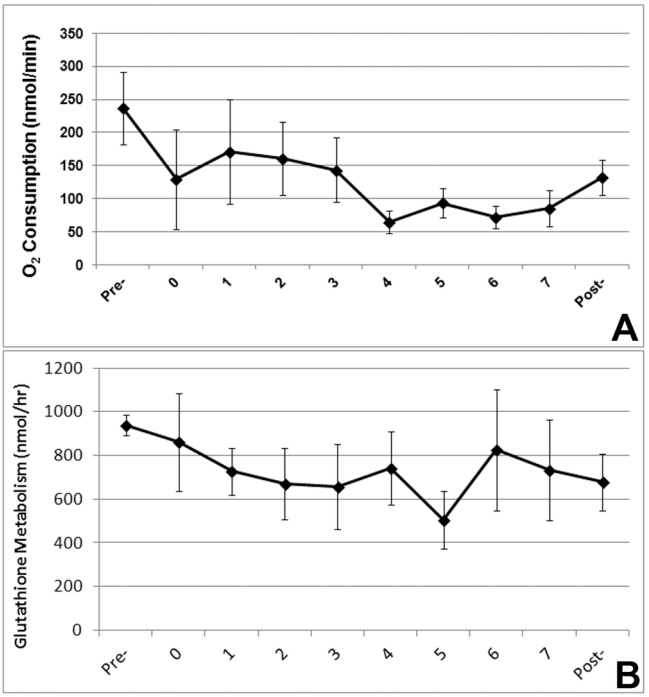

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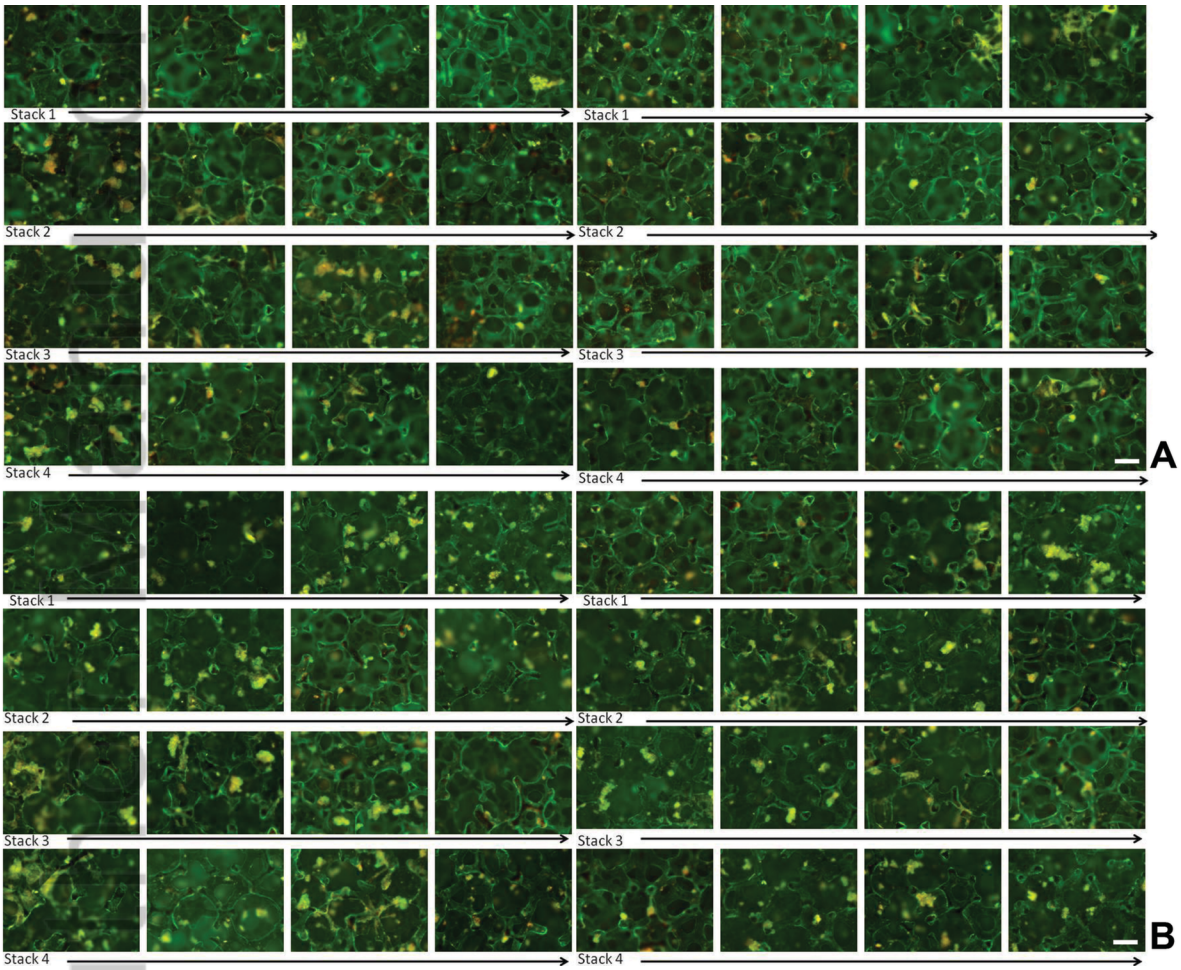

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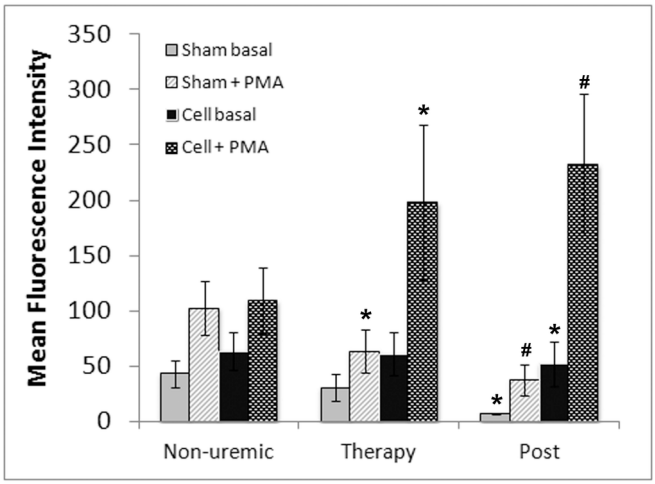

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