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TITLE: Resuscitation Strategies for Burn Injuries Sustained in Austere Environments to Improve Renal Perfusion and Function

PRINCIPAL INVESTIGATOR: David Burmeister

RECIPIENT: The Geneva Foundation
Tacoma, WA 98402

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14. ABSTRACT Our overall hypothesis is that oral or intravenous resuscitation results in distinct improvements in burn-induced SIRS and AKI. Specifically, while oral resuscitation (i.e., drinking) helps in reducing SIRS, MOD and AKI post-burn injury, we predict it will not be as effective as the gold standard i.v. fluid resuscitation which may relate to fluid volume requirements that cannot be met orally. Moreover, we hypothesize that i.v. blood products (e.g., fresh frozen plasma) will improve organ perfusion and outcomes when compared to crystalloids, and thus reduce total fluid requirements. Resuscitation strategies will vary in ameliorating burn induced renal perfusion and dysfunction because of a differential effect on circulating cytokines and granulocytes. Subsequently, markers and byproducts of oxidative stress will increase as renal perfusion decreases. Information from the studies described in this proposal will elucidate what effect low volume post-burn resuscitation strategies have on the mechanisms of oxidative stress and systemic and local inflammation. This will not only provide information on the ensuing SIRS, MOD, and AKI, but also allow for future testing of therapies to modulate these mechanisms. The ultimate goal is to improve outcomes after extensive burn in austere environments where large volumes of fluid are not available and the casualty is delayed in transport to a treatment facility.					
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INTRODUCTION:

Our overall hypothesis is that oral or intravenous resuscitation results in distinct improvements in burn-induced SIRS and AKI. Specifically, while oral resuscitation (i.e., drinking) helps in reducing SIRS, MOD and AKI post-burn injury, we predict it will not be as effective as the gold standard IV fluid resuscitation which may relate to fluid volume requirements that cannot be met orally. Moreover, we hypothesize that IV blood products (e.g., fresh frozen plasma) will improve organ perfusion and outcomes when compared to crystalloids, and thus reduce total fluid requirements. Resuscitation strategies will vary in ameliorating burn induced renal perfusion and dysfunction because of a differential effect on circulating cytokines and granulocytes. Subsequently, markers and byproducts of oxidative stress will increase as renal perfusion decreases. Information from the studies described in this proposal will elucidate what effect low volume post-burn resuscitation strategies have on the mechanisms of oxidative stress and systemic and local inflammation. This will not only provide information on the ensuing SIRS, MOD, and AKI, but also allow for future testing of therapies to modulate these mechanisms. The ultimate goal is to improve outcomes after extensive burn in austere environments where large volumes of fluid are not available and the casualty is delayed in transport to a treatment facility.

KEYWORDS:

Burn, prolonged field care, enteral, rehydration salts, intravenous, resuscitation, swine models, crystalloid, colloid, third spacing

ACCOMPLISHMENTS:

The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

Specific Aim 1: Determine the effectiveness of gastroenteral resuscitation in mitigating SIRS, MOD and AKI. (0-10 months)

- Objective 1a: Identify the effect of gastroenteral resuscitation on renal perfusion and AKI. (0-9 months) Large TBSA contact burn wound will be created using 9x15cm brass probes heated to 100°C on the dorsum, flanks, and hind limbs dorsum of Yorkshire pigs and treated with current standard of care wound dressings. Burn-induced SIRS will be characterized through routine blood collection by standard physiological parameters. Renal perfusion will be quantified via contrast enhanced CT angiography. AKI will be identified with blood chemistry analysis (e.g., creatinine) and urinalysis. By definition, all of these procedures are performed concurrently with animal experiments.
- Objective 1b: Identify the effect of gastroenteral resuscitation on systemic and local inflammation. (6-10 months) Serum and tissue biopsies collected at different time points will be analyzed for pro- and anti-inflammatory cytokines using ELISA techniques. Additionally, immunohistochemical techniques will be used to identify infiltrating immune cell populations, and to elucidate MOD in biopsies of kidneys, lungs, liver and intestine taken upon euthanasia.

Specific Aim 2: Determine the effectiveness of limited volume i.v. resuscitation for mitigating SIRS, MOD and AKI.

- Objective 2a: Compare the effects of instillation of lactated Ringer's (LR) as calculated by the modified Brooke Formula, versus a limited resuscitation volume paradigm. (10-21 months) Implanted jugular catheters will be used to administer 2 different volumes of LR to explore the consequences of limited volume capabilities likely found in prolonged field care scenarios. Outcomes will be similar to Aim 1 to examine: AKI via blood chemistry, urinalysis, and CT-angiography; SIRS via physiological parameters, blood cell counts, and cytokine (ELISA) analysis; and MOD via blood chemistry and histopathology. As such, results will be directly comparable to those found in Aim 1.
- Objective 2b: Compare the effects of LR with 2 different colloids: 5% albumin and fresh frozen plasma (FFP) on SIRS, MOD, and AKI. (13-21 months) Implanted jugular catheters will be used to deliver limited volumes of FFP and albumin. The volume used corresponds to low-volume resuscitation capabilities in prolonged field care scenarios (approximately 2 units/70kg patient/day). Again, the same volume is used in objective 2a, making the results directly comparable.

What was accomplished under these goals?

Objective 1a:

We have met the goals from this objective and have completed experiments for examining oral resuscitation (water deprivation, ad libitum access to water, ORS at 70mL/kg/d, and ORS at 15mL/kg/d. In the past year, a manuscript detailing these results has been finalized in the journal PLoS One (PLoS One. 2018 May 2; 13(5):e0195615). This manuscript (included as an appendix) delineated renal function in the form of creatinine and glomerular filtration rate, disclosed contents of urine excretion (e.g., protein, electrolytes, etc.), and reported the results of the CT-enhanced angiographies. This data has also been used as a baseline comparator for IV fluid experiments describe in Objective 2, which has been presented at conferences such as the Society for Critical Care Medicine (see below). In short, we have completed our objective in establishing the resuscitative importance of drinking fluids for improving kidney function post-burn.

Objective 1b:

We have continued analysis of mechanistic experiments examining the interplay of local and systemic inflammation and pathophysiology. For example, circulating cytokines (included in Gomez et al., PLoS One) were elevated in the absence of sufficient enteral and IV fluid. Additionally, we have begun to examine the impact of endotheliopathy post burn (Figure 1). Specifically, burn-induced increases in Syndecan-1 circulation are not dependent on enteral fluids and are transient in that they return back to baseline levels by 48 hours in all groups except for low-volume ORS. This information is currently being compiled for submission later this FY.

In terms of inflammatory cells, we are preparing a manuscript for submission on the state of splenic and circulating immune cells. Figure 2 shows information revealing that IV fluids (but not enteral fluids) significantly reduce the amount of neutrophils within circulation and in the spleen. Similar results are seen in monocytes but not for lymphocytes. Additionally, in the spirit of efficiency, we are actively pursuing extracting extra information from collected tissues from the first aim. For example, in the last year we have published a manuscript from the adrenal tissues of these animals in the Journal of Burn Care and Research (2018 Aug 17; 39(5):652-660),

which was focused on the adrenal response to burn, which was related to this objective, but not explicitly stated in the original SOW.

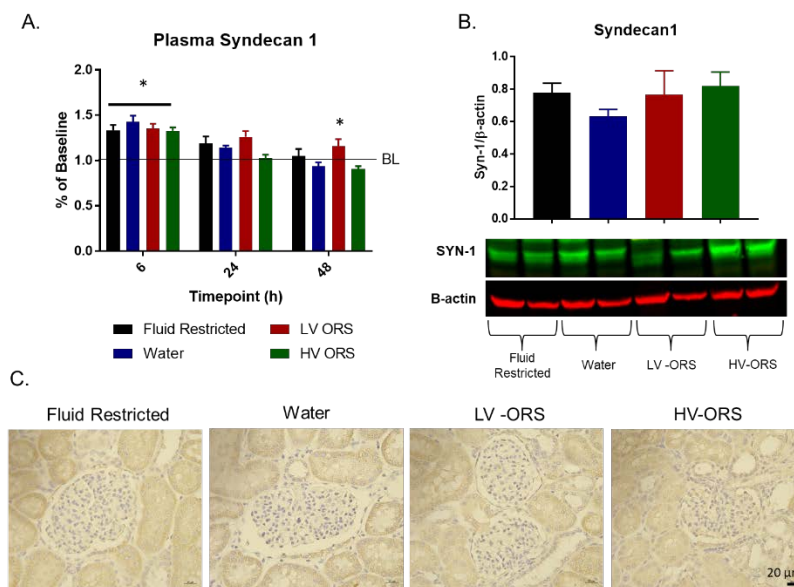


Figure 1. (A) Plasma syndecan levels increase post-burn regardless of enteral fluid levels. Additionally, renal levels of syndecan revealed via Western Blot (B) and Immunohistochemistry (C) also show no changes amongst the groups.

Taken together, these results indicate that enteral fluids may be a viable option for resuscitation of burn patients. From a safety standpoint, there were no adverse events (e.g., ileus) in our animal experiments, and these fluids were able to reverse acute kidney injury seen after 40% TBSA contact burn. While this method of delivering fluids is very simple and feasible in prolonged field care scenarios, it also may prove to be of benefit along with intravenous fluids as part of definitive clinical care. This is especially true when considering the shortage of IV fluids seen in the past year because of hurricane-based destruction of manufacturing facilities. As such, one of the most exciting accomplishments emanating from these conclusions is a standardization of an SOP to employ enteral fluids within USAISR's burn center. This SOP has been shared on the Department of Health and Human Services website, as a resource during mass casualty or other such events.

Objective 2a:

We have completed animal experiments from this Aim and have incorporated some of the circulating biomarkers in comparative reports with those in Aim 1. In terms of IV fluids, similar reversal of AKI was seen when compared with enteral fluids (i.e., non-inferiority). However, what was seen was significant third spacing in the subcutaneous space which was associated with higher weight gain with fluid levels approaching 2ml/kg/%TBSA. Analyses of tissues from these experiments have started very recently, with expected results in the no cost extension period. Another example of this is from the intestinal tissue, which is being analyzed for its relationship to the gut microbiome, which was analyzed with non-invasive fecal swabs.

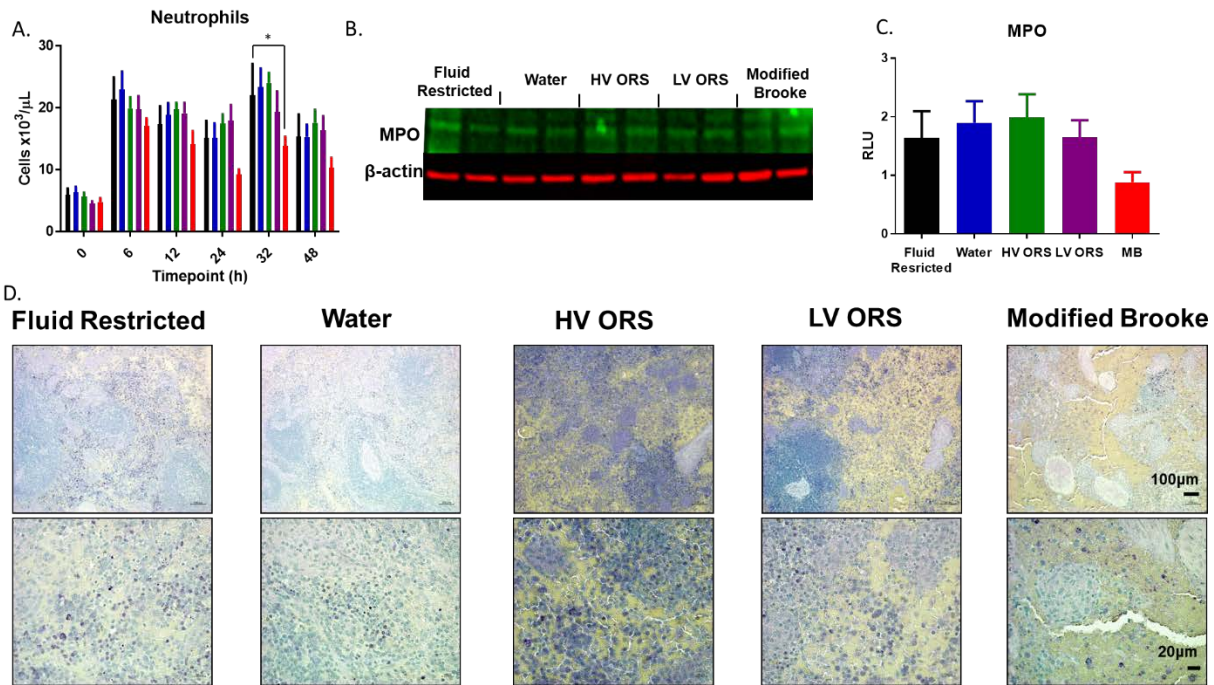


Figure 2. Circulating (A) and splenic tissue level (B-D) neutrophils are reduced in the presence of IV, but not enteral, fluids.

Objective 2b:

Animal experiments for this objective have very recently been completed. Analysis of the biochemistry and collected tissues has begun but is not currently far enough along to make specific conclusions. Initial broad interpretation of the data suggests that early use of albumin may have detrimental effects on the liver. However, full analysis of the ex vivo experiments examining the tissues and plasma from these animals will be needed to make definitive conclusions.

Objectives 1 and 2: We have submitted a no-cost extension for this contract to continue with ex vivo assays on collected organs and plasma from both aims.

What opportunities for training and professional development has the project provided?

While this project was not intended to specifically provide training or professional development, it has served as a platform for Dr. Burmeister to establish his laboratory, to include mentorship of two postdoctoral fellows who are involved with this project. One of these individuals has been promoted to Staff Scientist within the last year due to her hard work ethic and promise for influencing military medicine. This includes design of *ex vivo* experiments and writing/presentation of abstracts. Additionally, tissue and experiments from this project served as the basis for 4 summer student projects within the last 2 years. Specific presentations have been done at national conferences including Shock, Experimental Biology, American Burn Association, and Military Health Research Symposium.

How were the results disseminated to communities of interest?

Results were disseminated to the scientific community in the form of manuscript and oral presentations (see below). The burn center was briefed with internal updates, and creation of an SOP for its employment in the clinic.

What do you plan to do during the next reporting period to accomplish the goals?

The next reporting period will include: 1- A survey of the burn community about the use of enteral fluids, 2- Histological analyses, 3- Analysis of protein levels, and 4- Manuscript submissions

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The finding and results from this project indicate that enteral fluids may be of benefit, and it is anticipated that studies into the types and volumes of these fluids will be pursued. Ultimately, this strategy may eventually be employed routinely for the resuscitation of burn patients.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

As mentioned earlier, these results have been communicated to the burn center. Discussions with Dr. Cancio at the USAISR burn center have led to an SOP which has also been shared with the DHS Assistant Secretary of Preparedness and Readiness (See Appendix). The best way to capture the efficacy of these fluids within the clinic currently under discussion.

What was the impact on society beyond science and technology?

If the aforementioned clinical study being planned proves to corroborate the animal studies performed thus far, then it is possible that burn centers across the country/world will employ the use of enteral fluids. This would have far-reaching effects, especially in economically disadvantaged areas of the world, or in resource-poor environments (e.g., mass casualty scenarios, prolonged field care).

CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

In Q1, contracting problems with the veterinary staff prevented a swine shipment on November 17, 2016 (see attached MFR). However, because our animal protocol was approved ahead of schedule, this did not impede our progress as stated in the SoW.

Changes that had a significant impact on expenditures

A no-cost extension was requested to allow for more tissue processing time.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

PRODUCTS:

List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”

- **Publications, conference papers, and presentations**

Journal publications.

Burmeister DM, Little JS, Gomez BI, Gurney J, Chao T, Cancio L, Kramer GK, Dubick MA. Enteral Resuscitation Following Burn Injury in Resource-Poor Environments: Considerations Including Operational Advantages and Palatability. Under review, JSOM.

Burmeister DM, McIntyre MK, Montgomery RK, Gomez BI, Dubick MA. Isolation and Characterization of Multipotent CD24+CD133+ Cells from the Renal Papilla of Swine. Front Med. 2018 Sep19; 5:250. , published, yes.

Gomez BI, He C, Chao T, Dubick MA, Burmeister DM. Effect of Intravenous Fluid Volumes on the Adrenal Glucocorticoid Response after Burn Injury in Swine. J Burn Care Res. 2018 Aug 17; 39(5):652-660, published, yes.

Gomez BI, McIntyre MK, Gurney JM, Chung KK, Cancio LC, Dubick MA, Burmeister DM. Enteral Resuscitation with Oral Rehydration Solution to Reduce Acute Kidney Injury in Burn Victims: Evidence from a Porcine Model. PLoS One. 2018 May 2; 13(5):e0195615. Published, yes.

Burmeister DM, Gomez B, Dubick MA. Molecular mechanisms of trauma-induced Acute Kidney Injury: Inflammatory and metabolic insights from animal models. Biochim Biophys Acta. 2017 Oct; 1863(10 Pt B): 2661-2671. Published, yes.

Books or other non-periodical, one-time publications.

Nothing to Report.

Other publications, conference papers, and presentations.

Three abstracts were also published in conference proceedings: 5 abstracts have been part of official publications as well, in Federation of American Societies for Experimental Biology (FASEB), Society for Critical Care Medicine, and Shock.

1. DM Burmeister, BI Gómez, T Chao, LC Cancio, MA Dubick. 402 Enteral Resuscitation Shows Similar Efficacy to IV Resuscitation in a Porcine 40% TBSA Contact Model. Journal of Burn Care & Research 39 (suppl_1), S172-S172.
2. BI Gómez, C He, T Chao, MA Dubick, DM Burmeister. 113 Effect of Intravenous Fluid Resuscitation Volumes on the Adrenal Response in Burn Injury in Swine. Journal of Burn Care & Research 39 (suppl_1), S62-S62.

3. T Chao, BI Gomez, TC Heard, MA Dubick, DM Burmeister. 413 Altered Renal and Cardiac Mitochondrial Activity After 40% TBSA in a Swine Model. Journal of Burn Care & Research 39 (suppl_1), S178-S178.
4. BI Gómez, BK Harrington, T Chao, JS Little, TC Heard, MA Dubick, DM Burmeister. 229 Enteral Fluid Resuscitation Alters Splenic Function and Leukocyte Populations Post-Burn in Swine. Journal of Burn Care & Research 39 (suppl_1), S82-S82.
5. DM Burmeister, BI Gomez, T Chao, L Cancio, M Dubick. 832: Enteral Resuscitation Of Moderate Burns Shows Similar Efficacy To IV Resuscitation In A Swine Model. Critical Care Medicine 46 (1), 400.
6. BI Gomez, T Chao, MA Dubick, DM Burmeister. 8 Limited Volume Lactated Ringer's and Plasma-lyte are Comparable for IV Resuscitation in a Pig Burn Model. Shock 49, 43.
7. T Chao, BI Gomez, TC Heard, MA Dubick, DM Burmeister. 9 Fluid Resuscitation on Cardiac Mitochondrial Function in Severely Burned Swine. Shock 49, 43-44.

Three abstracts were presented at the 2018 Military Health System Research Symposium, the first as an oral presentation, and the second two as posters.

1. Tony Chao, Ph.D., Belinda Gómez, Ph.D., Tiffany Heard, SPC Joshua Little, Michael Dubick, Ph.D., David Burmeister, Ph.D. Fluid Resuscitation on Cardiac Mitochondrial Function in Severely Burned Swine. Military Health System Research Symposium, Kissimmee, FL, August 2018.
2. Burmeister, DM, Bynum, J, Little, JS, Wu X, Gomez, BI, Chao, T, Gurney, JM, Darlington, D, Dubick, MA. The Effect of IV Resuscitation on Coagulation and Platelet Aggregation After 40% TBSA Burns in Swine. Military Health System Research Symposium, Kissimmee, FL, August 2018.
3. Belinda I. Gómez, PhD, Tony Chao, PhD, SPC Joshua S. Little, Michael A. Dubick, PhD, David M. Burmeister, PhD. Limited Volume Lactated Ringer's and Plasma-Lyte are Comparable for IV Resuscitation in a Swine Burn Model. Military Health System Research Symposium, Kissimmee, FL, August 2018.

Website(s) or other Internet site(s)

<https://asprtracie.hhs.gov/technical-resources/28/burns/27>
<http://ameriburn.org/quality-care/mass-casualty/oral-fluid-resuscitation/>

• Technologies or techniques

Nothing to Report.

• Inventions, patent applications, and/or licenses

Nothing to Report.

• Other Products

Nothing to Report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: David Burmeister
Project Role: PI
Nearest person month worked: 3
Contribution to Project: Dr. Burmeister is providing technical oversight and leadership of the protocol. Specifically, he will oversee regulatory approval, supervise data collection and analysis, and coordinate team meetings to review planning and execution of the study.

Name: Belinda Gomez
Project Role: Postdoctoral Fellow
Nearest person month worked: 3
Contribution to Project: Dr. Gomez assisted with animal procedures and processed blood/tissue samples.

Name: Tony Chao
Project Role: Postdoctoral Fellow
Nearest person month worked: 1.2
Contribution to Project: Dr. Chao assisted with animal procedures and processed blood/tissue samples.

Name: Tiffany Heard
Project Role: Research Lab Technician III
Nearest person month worked: 12
Contribution to Project: Tiffany is learning assays that examine mitochondria function.

Name: Joshua Little
Project Role: Private First Class
Nearest person month worked: 1.2
Contribution to Project: Upon availability, PFC Little runs blood Vacutainer tubes to our biochemistry core lab, and aliquots plasma for later analysis.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

SPECIAL REPORTING REQUIREMENTS

Quad chart: Attached

APPENDICES

1. Burmeister DM, McIntyre MK, Montgomery RK, Gomez BI, Dubick MA. Isolation and Characterization of Multipotent CD24+CD133+ Cells from the Renal Papilla of Swine. *Front Med*. 2018 Sep19; 5:250. Published, yes.
2. Gomez BI, He C, Chao T, Dubick MA, Burmeister DM. Effect of Intravenous Fluid Volumes on the Adrenal Glucocorticoid Response after Burn Injury in Swine. *J Burn Care Res*. 2018 Aug 17; 39(5):652-660. Published, yes.
3. Gomez BI, McIntyre MK, Gurney JM, Chung KK, Cancio LC, Dubick MA, Burmeister DM. Enteral Resuscitation with Oral Rehydration Solution to Reduce Acute Kidney Injury in Burn Victims: Evidence from a Porcine Model. *PLoS One*. 2018 May 2; 13(5):e0195615. Published, yes.
4. Burmeister DM, Gomez B, Dubick MA. Molecular mechanisms of trauma-induced Acute Kidney Injury: Inflammatory and metabolic insights from animal models. *Biochim Biophys Acta*. 2017 Oct; 1863(10 Pt B): 2661-2671. Published, yes.



Isolation and Characterization of Multipotent CD24+ Cells From the Renal Papilla of Swine

David M. Burmeister*, Matthew K. McIntyre, Robbie K. Montgomery, Belinda I. Gómez and Michael A. Dubick

Damage Control Resuscitation, United States Army Institute of Surgical Research, San Antonio, TX, United States

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Edited by:

Maik Gollasch,
Charité Universitätsmedizin Berlin,
Germany

Reviewed by:

Björn Hegner,
Vivantes Humboldt-Klinikum, Germany
Jianliang Jin,
Nanjing Medical University, China

*Correspondence:

David M. Burmeister
david.m.burmeister3.civ@mail.mil

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Over 100,000 patients in the United States are currently waiting for a kidney transplant. With just over 10,000 cadaveric kidneys transplanted annually, it is of the utmost importance to optimize kidney viability upon transplantation. One exciting avenue may be xenotransplantation, which has rejuvenated interest after advanced gene editing techniques have been successfully used in swine. Simultaneously, acute kidney injury (AKI) is associated with high morbidity and mortality and currently lacks effective treatment. Animal models have been used extensively to address both of these issues, with recent emphasis on renal progenitor cells (RPCs). Due to anatomical similarities to humans we aimed to examine progenitor cells from the renal papillae of swine kidneys. To do this, RPCs were dissected from the renal papillae of healthy swine. Cell surface marker expression, proliferation, and differentiation of the RPCs were tested *in vitro*. Additionally, a mixed lymphocyte reaction was performed to examine immunomodulatory properties. RPCs displayed spindle shaped morphology with limited self-renewing capacity. Isolated RPCs were positive for CD24 and CD133 at early passages, but lost expression with subsequent passaging. Similarly, RPCs displayed myogenic, osteogenic, and adipogenic differentiation capacities at passage 2, but largely lost this by passage 6. Lastly, direct contact of RPCs with human lymphocytes increased release of IL6 and IL8. Taken together, RPCs from the papilla of porcine kidneys display transient stem cell properties that are lost with passaging, and either represent multiple types of progenitor cells, or a multipotent progenitor population. In instances of ischemic insult, augmentation of/with RPCs may potentiate regenerative properties of the kidney. While the use of swine for transplantation and ischemia studies confers obvious advantages, the populations of different progenitor cell populations within pig kidneys warrants further investigation. Ultimately, while gene editing techniques enhance the potential for xenotransplantation of organs or cells, the ultimate success of this strategy may be determined by the (dis)similarities of RPCs from different species.

Keywords: stem cells, papillae, pig models, transplantation, progenitor cells, swine, kidney

INTRODUCTION

While roughly 17,000 kidney transplants are performed annually in the United States, over 100,000 Americans are currently waiting for a kidney transplant (1). With such a paucity of donor kidneys, there is a great need to maximize organ preservation in order to prevent rejection and improve long term outcomes post-transplantation (2). To realize these optimal conditions in the face of precious few human kidneys, both small (3) and large (4) animal models have provided valuable insight. Moreover, the potential for filling this need with xenotransplantation has been made even more possible with advanced gene editing techniques (5) and other burgeoning technologies that render clinical trials utilizing pig kidneys closer than ever (6).

Separately, acute kidney injury (AKI) disrupts the kidneys' blood filtering function and increases mortality, hospital length of stay, and hospital costs (7). Molecular mechanisms driving AKI have also been informed by a variety of animal models from chemical induction to trauma (8, 9). However, there remains a growing need to address the lack of effective treatments for AKI. More recent advances in stem cell biology have led to the examination of various cell populations in animal models of renal disease. For example, a variety of cell types have been used to examine therapeutic efficacy in both AKI and renal transplantation models (10–12).

Alternatively, it has been shown that renal recovery following ischemia-reperfusion injury is influenced by a population of intrinsic renal cells rather than extra-renal mesenchymal cells (13, 14). Indeed, there have been several studies that have attempted to localize the renal stem cell niche via such methods as BrdU label retention. These studies have resulted in different claims of renal stem cells from locations such as proximal tubules, renal papillae, parietal epithelial cells, glomeruli, or Bowman's capsule (15–20). Recent consensus seems to have been reached that regeneration of the tubular epithelium occurs from cells that originate in the tubules. However, debate still ensues as to whether or not these cells are resident stem cells in the proximal tubules, or if all terminally differentiated tubular epithelial cells have the ability to dedifferentiate and proliferate to repopulate the tubular epithelium (21, 22). It is likely that different progenitor cell populations are involved with different reparative processes depending on the etiology of renal dysfunction.

Along these lines, the controversy over the existence and localization of these cells has recently been addressed by the theory that multiple populations of progenitor cells exist within the adult kidney (23). This notion was further evidenced in a recent report by Oliver et al. which used genetic lineage tracing of *Zfyve27* to show that different locations in the kidney are populated by different progenitor cell sets (24). One such location for renal progenitor cells (RPCs) is the renal papilla (19), which has been shown to yield such cells in a recent study in rodents (25). While many studies have been carried out in small animals, further pre-clinical studies in large animals are needed (26). In particular, the kidneys of domestic swine have several characteristics that give certain advantages for comparison to human renal (patho) physiology. Swine kidneys are pyramidal/multi-lobular with similar vasculature to

human kidneys (4). Kidney and body size, as well as renal biochemical markers creatinine and blood urea nitrogen (BUN), are also comparable between pigs and humans. To examine this progenitor cell population in pigs, we isolated renal papilla from porcine kidneys and examined their: proliferative ability; capacity to differentiate into multiple lineages; cell surface expression of CD24 and CD133 and; immunomodulatory properties. We find that these cells are heterogeneous in nature, have limited self-renewing capacity and stem-cell like properties that are lost with subsequent passaging.

MATERIALS AND METHODS

Animals

Seven Yorkshire pigs (Midwest Research Swine, Gibbon, MN) weighing 68.0 ± 4.9 kg were used in this study. To ensure health of the animal and the kidney, blood samples were taken prior to euthanasia for analysis of blood chemistry and complete blood count (CBC). All procedures were approved by the Regulatory Research Compliance Division of the US Army Institute of Surgical Research (USAISR). Specifically, a tissue sharing request to the Animal Care and Use Committee from USAISR was completed. This research was conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals, National Research Council. The facility's Institutional Animal Care and Use Committee approved all research conducted in this study. The facility where this research was conducted is fully accredited by AAALAC International. For protein, creatinine, and BUN measurement, blood was collected into a BD Vacutainer tube with Lithium Heparin (Becton Dickinson and Company, Franklin Lakes, NJ) and centrifuged at 1,200 g for 10 min. Samples were analyzed on a Siemens Dimension® Xpand™ Plus clinical Chemistry system. For CBC analysis, specimens were collected in a BD Vacutainer tube containing K2 EDTA (Becton Dickinson and Company, Franklin Lakes, NJ) and analyzed with the Abbott Cell-Dyn® 3700 system.

RPC Isolation

Animals were euthanized with pentobarbital sodium. Using sterile technique, kidneys were excised, and placed in Hanks Balanced Salt Solution (HBSS) (Gibco, Gaithersburg, MD) immediately post-mortem for isolation of the renal papilla as previously described (27) and shown in **Figure 1**. Briefly, the renal capsule was removed and a longitudinal incision along the coronal axis was made to identify and remove the renal papillae. Papillae were washed with HBSS and minced, before being digested in Collagenase type II (Gibco, 2 mg/mL in HBSS) for 30 min at 37°C. Samples were centrifuged at 1,900 rpm for 10 min and the pellet was re-suspended in Mesenpro RS™ growth media (Gibco) containing supplement, l-glutamine (Gibco), and 1–2% antibiotics/antimycotics (AB/AM, Gibco). Cells were plated on T-75 flasks (Corning, Corning, NY) and incubated at 37°C with 5% CO₂. Non-adherent cells were removed at day 2 and media was changed every 2–3 days. The remaining adherent cells were defined as RPC and allowed to reach 80% confluency after which

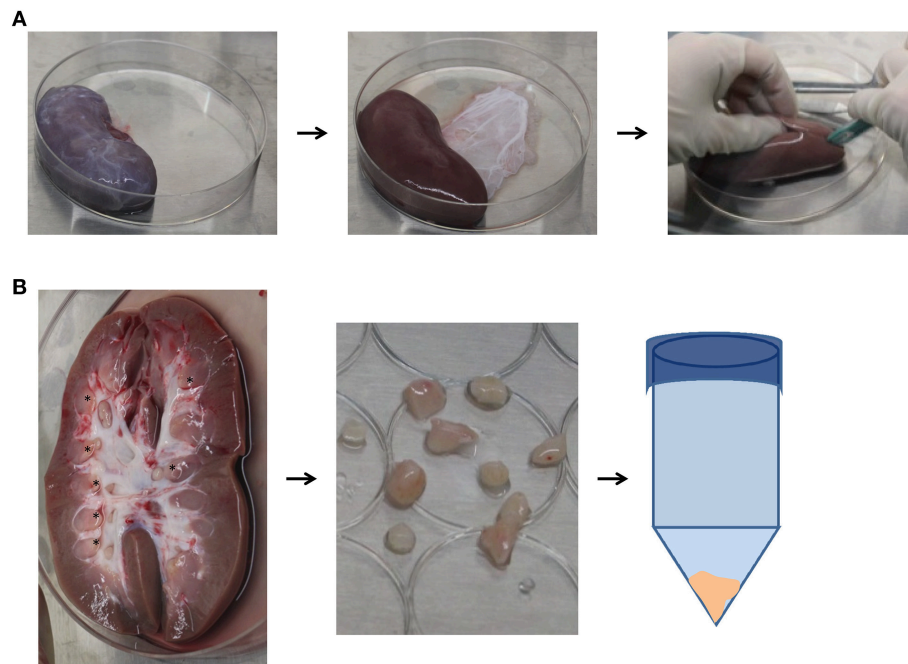


FIGURE 1 | Isolation procedure for papillary renal progenitor cells. **(A)** Sterile blunt dissection of excised porcine kidneys was done via removal of the renal capsule, and a subsequent coronal incision. **(B)** Visible papillary tips (asterisks) were cut away from the remainder of the kidney and minced with scissors, where they were then incubated in 2mg/mL collagenase at 37°C for 30 min. After enzymatic digestion, papillae were centrifuged, the supernatant was discarded, and cell pellet plated.

they were passaged with 0.25% Trypsin/EDTA (Gibco) for 5 min before neutralization with Mesenpro RSTM growth media.

Proliferation

At passages 2 and 6, 1×10^4 RPCs were plated into triplicate wells of a 96-well plate and grown in Mesenpro RSTM media with supplements listed above. At days 1, 2, 3, and 7 post-plating, media was removed and replaced with 4% paraformaldehyde (PFA) until all cells were fixed, then plates were stored at -80°C until analysis. A standard curve was generated on each plate by seeding increasing amounts of cells in triplicate, allowing 4 h for adherence and then fixing cells with 4% paraformaldehyde (PFA) and subsequently freezing. CyQUANT Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR) was performed according to manufacturer's protocol for determining the density of cells.

Immunofluorescence Microscopy

At passages 2 and 6, 5×10^3 RPCs were seeded on a 96-well plate in triplicate and allowed to adhere for 4 h in growth media. Cells were fixed with 4% PFA in HBSS for 15 min and subsequently washed with HBSS. Cells were then blocked with 1% BSA (Sigma Aldrich, St. Louis, MO) with 0.1% Triton X-100 (Thermo Fisher, Waltham, MA) for 1 h at RT. The following primary antibodies were used for immunocytochemistry: CD24 (Bioss, 1:100 dilution), CD133/Prom1 (Genway, GWB-MW170D, 1:50 dilution), NFATC-1 (LSBio, LS-C40633, 1:10 dilution), CD90 (BD Biosciences, 555595, 1:10 dilution), and CD45 (R & D Systems, FAB1430P, 1:10 dilution); all diluted in blocking buffer

for 3 h at room temperature. Cells were then washed, blocked again with BSA solution for 1 h, and subsequently incubated with either Donkey anti-rabbit Alexa Fluor 488 secondary antibodies (Thermo, 1:200 dilution) for 1 h at RT. Cells were again washed, and incubated with HBSS containing $0.5 \mu\text{g/mL}$ DAPI.

Flow Cytometry

At passages 2 and 6, 5×10^5 RPCs were collected as described above and re-suspended in $100 \mu\text{L}$ of HBSS. The same antibodies listed above for CD24 (1:100 dilution), CD133/Prom1 (1:20 dilution), and NFATC-1 (1:20 dilution) were conjugated with ZenonTM Alexa FluorTM 647 or 488 anti-rabbit IgG labeling kit according to manufacturer protocol. An unstained population of RPCs, and one incubated with the Zenon reagents served to set the size and fluorescence gating. After mixing, $100 \mu\text{L}$ of the entire solution was then added to cells and incubated in the dark for 1 h. Cells were then washed once and flow cytometry was performed using a BD FACSCanto (BD biosciences, San Jose, CA) by collecting 10,000 events/sample. Analysis was performed with BDFACS Diva software.

Adipogenic Differentiation

Adipogenic differentiation was performed as previously described for other mesenchymal stem cells (MSCs) (28). All chemicals were purchased from Sigma-Aldrich unless otherwise specified. Briefly, passage 2 and 6 RPCs were seeded on T-25 flasks at a density of 1×10^4 cells/cm² containing high glucose DMEM containing 10% FBS, Ab/Am, L-glutamine, 0.5 mM

Isobutyl-methyl xanthine, 200 μ M indomethacin, 10 μ M insulin, 1 μ M dexamethasone, and 10 μ M ciglitazone and incubated at 37°C supplemented with 5% CO₂. Media was changed every 2–3 days and cells were fixed with 4% PFA after 2 weeks. After all passages were collected, cells were washed with dH₂O and incubated with 60% isopropanol before incubation with Oil Red O solution.

Osteogenic Differentiation

Osteogenic differentiation was performed as previously described for other MSCs (28). Briefly, passage 2 and 6 RPCs were seeded on T-25 flasks at a density of 1×10^3 cells/cm² containing α -MEM containing 10% FBS, Ab/AM, L-glutamine, 50 μ M Ascorbate-2-phosphate, 10 mM β -glycerophosphate, 10 ng/mL bone morphogenic protein 2 (R&D Systems), and 0.1 μ M dexamethasone and incubated at 37°C supplemented with 5% CO₂. Media was changed every 2–3 days and cells were fixed with 4% PFA after 2 weeks, after which cells were washed with HBSS [no Ca⁺⁺]] and stained with Alizarin red solution (Millipore).

Myogenic Differentiation

Myogenic differentiation was performed as previously described for other MSCs (28). Briefly, passage 2 and 6 RPCs were seeded on T-25 flasks at a density of 2×10^3 cells/cm² that were previously coated with 10 μ L/cm² Matrigel (Corning) diluted 1:6 in myogenic differentiation media containing low glucose DMEM supplemented with 5% Horse serum, AB/AM, 50 μ M hydrocortisone, and 0.1 μ M dexamethasone and incubated at 37°C supplemented with 5% CO₂. Media was changed every 2–3 days and cells were fixed with 4% PFA after 2 weeks. After all passages were collected, cells were blocked with 1% BSA containing 0.1% Triton X-100 for 1 h, and incubated overnight at 4°C with anti-Myosin Heavy Chain antibody (R&D Systems) (diluted 1:50) in 2% goat serum. The following day cells were washed with PBS and blocked for 1 h in 5% goat serum and then incubated for 2 h at room temperature in Alexa-488 goat anti-mouse antibody (Life Technologies; 1:400) in 2% goat serum.

RPC Effect on Porcine PBMCs

To examine the direct and paracrine effects of RPCs on peripheral blood mononuclear cell (PBMC) cytokine production, a mixed lymphocyte reaction was performed. PBMCs were isolated from healthy donor pigs ($n = 4$) in duplicate using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) according to manufacturer protocol. Lymphocytes were washed with HBSS and re-suspended in RPMI-1640 medium (ATCC modification, Gibco) supplemented with 10% FBS (Gibco), and 1% AB/AM (Gibco). 2×10^5 PBMC were seeded on 24 well plates. In duplicate wells 2×10^5 passage 2 RPCs were then added either in the well directly, or into 0.4 μ m pore size polycarbonate transwell inserts, or control wells were made with no RPCs. To stimulate the PBMCs 5 μ g/mL of phytohemagglutinin-L (PHA) was added and the cells were allowed to incubate for 3 days at 37°C supplemented with 5% CO₂. Cell supernatant was collected and stored at –80°C until analysis. Supernatants were analyzed with a porcine-specific MILLIPLEX Cytokine/Chemokine magnetic

TABLE 1 | Various kidney function and leukocyte levels circulating in animals used for cell isolation.

Measure	Unit	Mean $\pm$ SEM
Creatinine	mg/dL	1.61 $\pm$ 0.09
Total protein	g/dL	6.68 $\pm$ 0.14
BUN	mg/dL	9.55 $\pm$ 0.60
Creatinine kinase	U/L	513.16 $\pm$ 78.21
Neutrophils	$\times 10^3/\mu$ l	3.19 $\pm$ 0.51
Lymphocytes	$\times 10^3/\mu$ l	6.72 $\pm$ 0.62
Monocytes	$\times 10^3/\mu$ l	0.31 $\pm$ 0.03
Eosinophils	$\times 10^3/\mu$ l	0.41 $\pm$ 0.06
Basophils	$\times 10^3/\mu$ l	0.05 $\pm$ 0.01

bead panel kit (EMD Millipore, PCYTMG-23K-13PX), which was performed according to manufacturer protocol.

Statistical Analysis

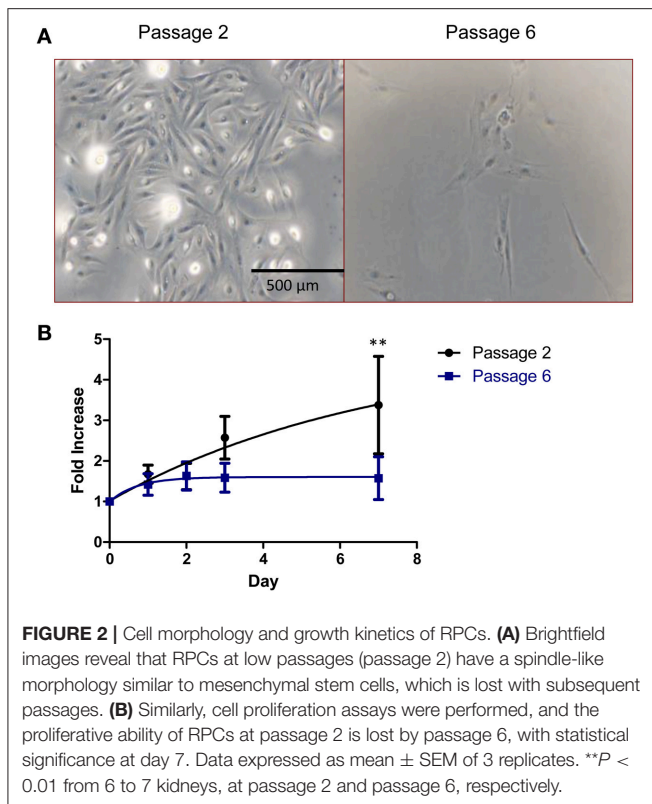
Statistical evaluations were performed using GraphPad Prism software (GraphPad Software, San Diego, CA). For the proliferation assay, a two-way ANOVA was performed to examine the effect of time and passage, with Bonferroni *post-hoc* testing. For the PBMC cytokine release, non-normally distributed data dictated that a Kruskal Wallis test with Dunn's Multiple Comparisons was employed. Technical replicates were averaged to produce a single value for biological replicates, which are expressed as the arithmetic mean $\pm$ SEM, and $p < 0.05$ were considered significant.

RESULTS

Morphology and Growth of Porcine RPCs

We isolated renal papillae from porcine kidneys immediately post-mortem under surgical sterile conditions (**Figure 1**). **Table 1** shows circulating biochemical values from the animal just before euthanasia. All animals displayed creatinine, BUN, total protein, and creatinine kinase values within the normal range for swine, indicating that renal function was not compromised. Moreover, the values of circulating white blood cells were also within normal range, indicating overall health of the animal (**Table 1**).

As shown in **Figure 2A**, isolated RPCs exhibited classic mesenchymal spindle shaped morphology and propensity to adhere to standard tissue culture plastic. At early passages (passage 2 shown in **Figure 2A**) RPC routinely expanded until harvesting/passaging at 80% confluence. However with subsequent passaging, it became apparent that the self-renewing capacity of porcine RPCs is limited by passage 6. Quantification of proliferative capacity is demonstrated in **Figure 2B** for cells at passage 2 and passage 6, wherein RPCs at the later passage failed to divide. Two-way ANOVA revealed a significant effect of time ($P = 0.0095$) and passage ($P = 0.0469$) on RPC proliferation. The fold increase in cells was significantly higher for passage 2 RPCs at day 7 ($p < 0.01$, $n = 6$ for passage 2, and $n = 7$ for passage 6), but not quite significant for day 3 ($P = 0.093$). There was some heterogeneity in the longevity of primary cultures despite consistent isolation protocols. Specifically, we



observed one population out of seven that continued to display a proliferative ability at passage 6, which is reflected in the variability.

Stem Cell Markers Present on RPCs

To examine whether RPCs expressed common renal “stem cell” population markers we performed immunocytochemistry and flow cytometry on CD24, CD133, and nuclear factor of activated T-cells 1 (NFATc1) (Figure 3). At passage 2, $96.3 \pm 1.1\%$ of isolated RPCs expressed CD24 staining which was confirmed with immunocytochemistry. However by passage 6, this percentage was drastically reduced to $23.8 \pm 11.2\%$, ($P = 0.0095$) with a variability reflecting heterogeneity in that only one isolate still expressed significant CD24. Immunocytochemical staining for CD133 proved to be strong in RPCs at both passages 2 and 6, although by passage 6 there were isolated areas of striated staining apparent. However, flow cytometry analysis with the same antibody did not corroborate this, likely due to the lack of antibody suitability for flow cytometry. A minor amount of NFATc1 ($6.85 \pm 0.93\%$, and $4.75 \pm 2.21\%$ at passage 2 and 6, respectively) was expressed as determined by flow cytometry. Expression of NFATc1 was also largely not seen with immunocytochemistry. Similarly, CD45 expression was low $14.55 \pm 1.97\%$, but higher than NFATc1. A certain degree of heterogeneity in stem cell marker expression was seen, as CD90 expression was $44.4 \pm 2.6\%$ and $47.2 \pm 6.0\%$ at passages 2 and 6, respectively.

Multipotent Differentiation of RPCs

To examine whether RPCs have the ability to differentiate into other cells of mesenchymal origin, we utilized previously established differentiation protocols as detailed in the Methods, to induce adipogenesis, osteogenesis, and myogenesis. As shown in Figure 4, isolated RPCs demonstrated multipotent ability in a passage-dependent manner. It is important to note that, at no point, did differentiation occur in all RPCs for any of these lineages, illustrating the heterogeneous phenotype of these cells. RPCs introduced to myogenic differentiation media began to express levels of myosin heavy chain after 2 weeks at passage 2, while RPCs at passage 6 did not have this ability. Similarly, the Alizarin Red staining seen with culturing passage 2 RPCs in osteogenic media was not apparent at passage 6. While passage 2 RPCs did show a limited ability for adipogenesis in that respective media, the drop off in this differentiation capacity from passage 2 to passage 6 was not as marked as with the other two lineages.

RPC's Effect on Mixed Lymphocyte Reactions

To elucidate the potential immunomodulatory properties of RPCs, we performed a mixed leukocyte reaction to examine both direct and paracrine effects of these cells on lymphocytes. As shown in Figure 5, we found a significant effect of RPCs on IL6 release ($P = 0.05$), wherein PHA-stimulated PBMCs released 16.25 ± 0.25 pg/mL in the absence of RPCs, 18.38 ± 0.38 pg/mL when introduced to indirect RPCs, and 19.01 ± 1.08 pg/mL when in direct contact with RPCs. A similar effect was seen with IL8 ($P = 0.025$) with values of 22.88 ± 2.35 , 75.19 ± 17.33 , and 102.4 ± 42.55 pg/mL being released with PBMCs alone, with indirect RPC contact, and with direct RPC contact, respectively. In contrast to this, we observed a slight decrease in IFN- γ release when RPCs were introduced to the culture; however this difference was not statistically significant.

DISCUSSION

With over 100,000 patients awaiting a kidney transplant, and another 3,000 added each month, there exists a great need to address more efficient methods of treating renal dysfunction (1). Tissue engineering and regenerative medicine technologies hold immense promise for addressing both AKI and chronic kidney disease (CKD). Recent studies have shown the feasibility of isolating RPCs from adult humans (29). Moreover, their documented (and somewhat surprising) therapeutic efficacy in animal models has generated much excitement for the use of RPCs in renal diseases. As swine models have several advantages in studying renal function, we aimed to determine the regenerative properties of a population of renal progenitors. The primary finding of the study presented herein is that cells isolated from the papillae of porcine kidneys represent a heterogeneous population with RPCs that have limited self-renewing and immunomodulatory capacities.

The limited proliferative ability of porcine RPCs does not preclude their potential use as therapies, as other cell populations or strategies could prove to increase RPC yield. The RPCs isolated

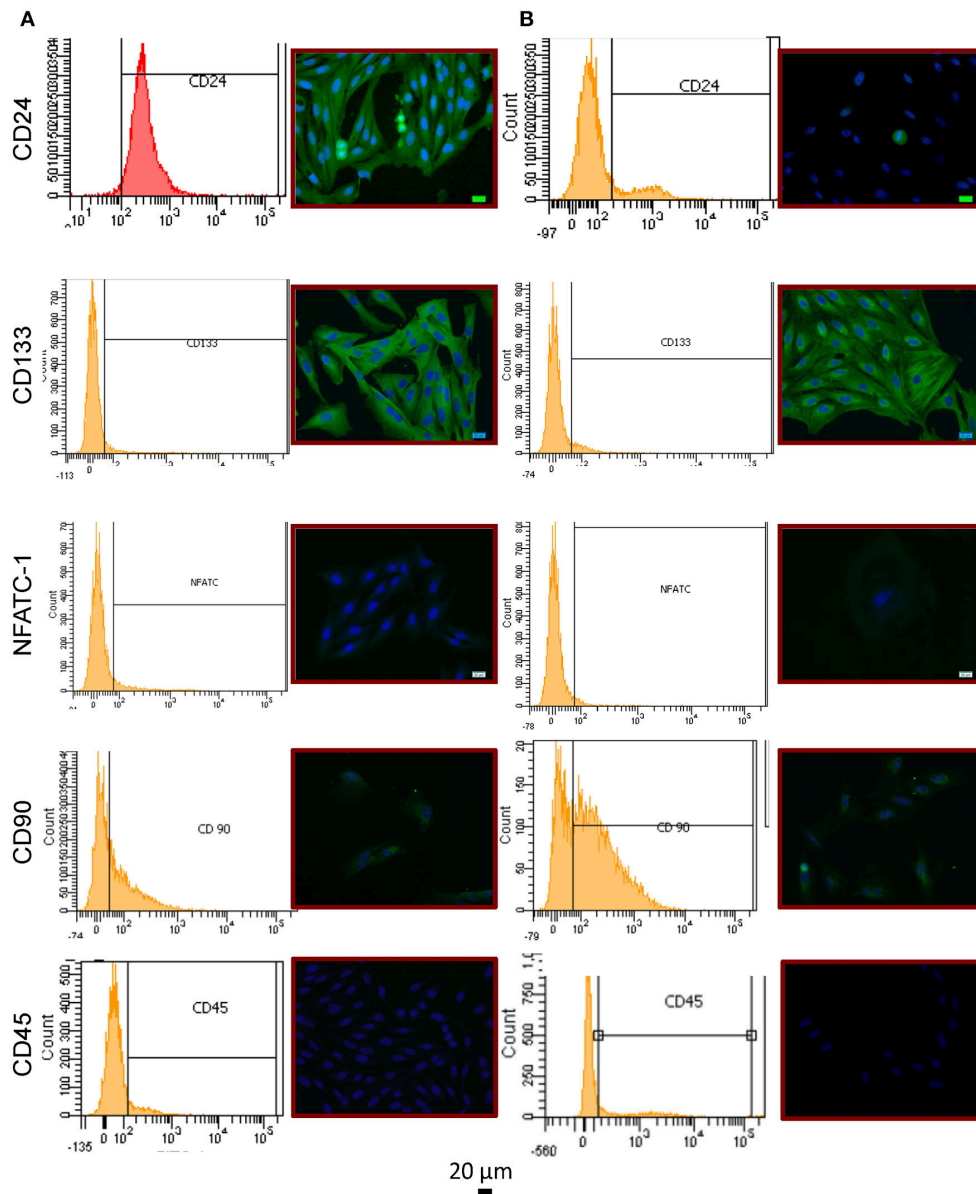


FIGURE 3 | Immunophenotype of RPCs **(A)** Passage 2 cells showed expression of CD24 (top) via immunocytochemistry as well as flow cytometry. While CD133 expression (middle) was strongly evident with immunocytochemistry, the same antibody was not able to detect expression via flow cytometry. CD90 was shown to be weakly expressed, and not in all cells. NFATc1 (middle row) and CD45 (bottom row) were shown not to be expressed with either method. **(B)** By passage 6, CD24 expression was drastically reduced.

herein were from the renal papillae, however other locations may produce a more robust cell line and growth. Bussolati et al. have described a population of RPCs from the cortex of human kidneys that also have a limited ability to proliferate (30). The cells in that study were not able to be passaged more than 7–9 times and exhibited a heterogeneity that we also encountered in the current study (Figure 2). However, populations of RPCs have also been isolated specifically from, for example, proximal (31) and distal (32) tubules, and Bowman's capsule (20) of adult human kidneys (23). Altogether, the limited yield of RPCs from

porcine renal papillae may make de- and re-cellularization efforts in porcine kidneys difficult, although worthwhile (33). However, RPCs examined herein were cultured on tissue culture plastic, and there remains the possibility of using substrates such as renal extracellular matrix to promote growth (34).

Despite the diversity in the localizations of different progenitor cells, the majority of these cells are reported to have surface marker expression of CD133 and CD24 (35). Resident CD133+CD24+ cells may play a key role in tubular repair following AKI due to their ability to differentiate into podocyte

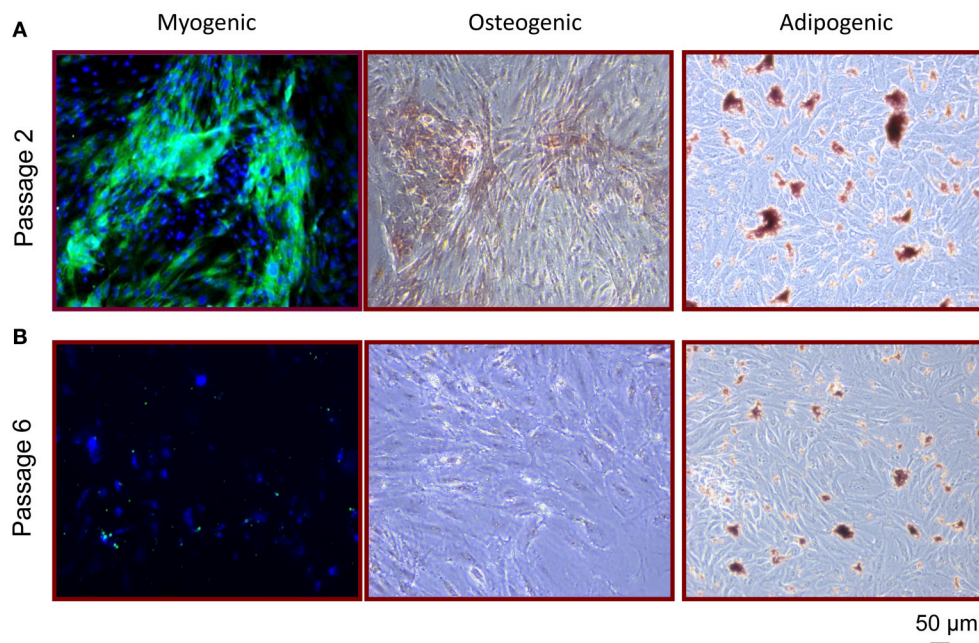


FIGURE 4 | Multipotent differentiation potential of RPCs. **(A)** At passage 2, RPCs were able to differentiate into myogenic (left), osteogenic (middle), and adipogenic (right) lineages, which was not as apparent at passage 6 **(B)**.

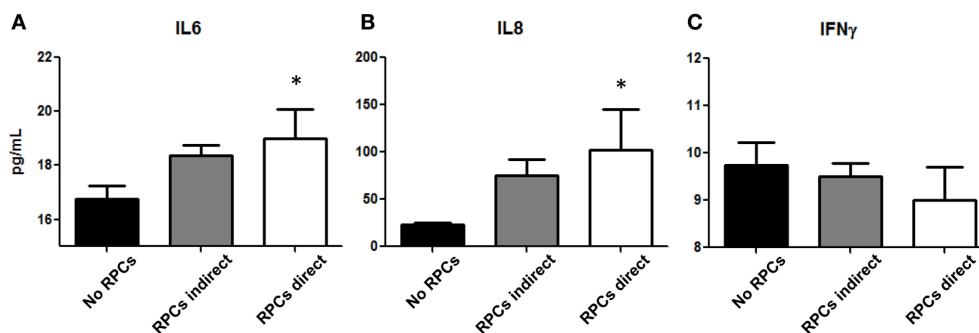


FIGURE 5 | RPCs effect on Peripheral Blood Mononuclear Cells. Cytokines IL-6 **(A)**, IL-8 **(B)**, and IFN γ **(C)** released from isolated lymphocytes from healthy swine are altered with the addition of RPCs. Both IL-6 and IL-8 (CXCL-8) are slightly increased when RPCs are placed into a well insert (gray bars), however they are significantly increased in media when RPCs are put into direct contact with PBMCs (white bars, * $P < 0.05$) when compared to PBMCs in the absence of RPCs (black bars). In contrast, there was no significant difference in IFN- γ release amongst the three groups. Data expressed as mean $\pm$ SEM of 2 replicates from 4 kidneys. * $p \leq 0.05$ from PBMCs in the absence of RPCs.

and tubular lineages (32). Additionally, the number of CD133+ cells is elevated in patients with renal pathologies (36). Isolation of CD24+CD133+ cells from normal human kidneys have been shown to differentiate into tubular, osteogenic, neuronal, and adipocyte cells *in vitro*, and also have the ability to regenerate tubular structures and attenuate kidney damage when injected into immunodeficient mice in acute renal failure (20). As such, these cells represent a viable treatment strategy for conditions of renal dysfunction (37).

Cell surface markers have been at the center of the conversation regarding the different locations of RPCs. While markers such as NFATc1 have been used to illustrate a progenitor

cell population from proximal tubules (17), CD24 is one of the consensus markers denoting RPCs (38). RPCs with dual expression of CD24 and CD133 have been shown to possess high regenerative capacity for the treatment of AKI (32, 37), and have the ability to regenerate into podocytes (39). Specifically, CD133+ RPCs cells from renal papilla have been shown to integrate into developing tubules (40). Moreover, CD133+ RPCs promote angiogenesis and erythropoietin production while reducing fibrosis (41). The RPCs in this study expressed CD24 in a passage-dependent manner, indicating senescence might reduce the therapeutic efficacy upon passaging (Figure 3). While flow cytometry did not demonstrate appreciable CD133

expression, the signal with the same antibody was strongly positive in immunocytochemistry, which we attribute to lack of antibody applicability for flow cytometry. This lack of porcine-specific reagents for specific analytical technology is currently a significant obstacle for expanded research into RPCs and/or renal diseases in swine. Moreover, by passage 6 the staining pattern in these cells include areas of a more striated pattern, raising the possibility of non-specific staining with this antibody and highlighting the heterogeneous nature of these cells.

As mentioned in the introduction, the location of the salient RPC population may depend on the underlying cause/etiology of renal dysfunction. For example, it has been shown after ischemia reperfusion injury that tubular regeneration occurs due to cells that are intrinsic to the tubular epithelium (42). Despite this, there remains significant controversy over the phenotype of these cells. One theory suggests that there are resident stem/progenitor cells (also termed “scattered tubular cells”) within the tubular epithelium, while another contends that each tubular epithelial cell has the ability to dedifferentiate and subsequently divide to repair the epithelium (21, 22). There is also evidence showing that tubular RPCs may contribute to tubular repair in the medulla, with the supposition that different regions of the kidney have their own progenitor cell pools (24). While cell sorting methods have been used for isolation of RPCs from these various locations, a recent report displayed a simple primary culture technique from the renal papillae of mice that resulted in a largely homogenous population of cells positive for stem cell markers such as CD90 and CD73 (25). Our efforts in the current study to recapitulate this process using kidneys from large animals resulted in a much more heterogeneous population with mixed positivity for stem cell markers.

The use of swine in kidney disease/transplantation research confers several advantages due to similarities with human kidneys (4). Pig kidneys have a multi-lobed structure similar to humans, with comparable renal size and vascularization. Swine also have similar body sizes which allow for the serial collection of blood samples or kidney biopsies. Pigs have been used in order to optimize the isolation of different RPC populations (43), and have also been used to examine the effectiveness of stem cells in kidney transplant survival (10). Moreover, knockout animals have produced successful xenotransplantation preclinical studies, raising the distinct possibility of renal transplantation from pigs to humans (5). With embryonic human and porcine renal precursors displaying similar ability for nephrogenesis (44), more research needs to be done on the role and efficacy of porcine RPCs.

The immunomodulatory properties of RPCs may eventually dictate their efficacy (or lack thereof) in AKI or other renal diseases. Although comparatively understudied compared to other stem cell types, a series of studies by Huang et al. found that kidney-derived stromal cells from mice have significant immunomodulatory activity (45, 46). The RPCs in that study stimulated bone marrow-derived dendritic cells that exhibited reduced stimulatory effects on CD4+ T cells, which was mediated through cytokine (i.e., IL6, IL1 β , and IL12) and vascular endothelial growth factor (VEGF) excretion. Dekel et al. also showed in mice that RPCs lack major histocompatibility complex

proteins which may aid in their ability to modulate CD8+ T cells (47). Unfortunately, there remains a paucity of studies that examine RPCs' effects on lymphocytes, especially among larger animal models. While bone marrow MSCs have been shown ameliorate aging in the kidney (27), another study showed a lack of efficacy for porcine bone marrow mesenchymal stem cells (MSC) in reducing AKI (48). This was driven, in part, by their low immunomodulatory activity, with porcine MSCs actually causing increased IL6 production. We found similar results in this study, wherein proinflammatory cytokines IL6 and IL8 were increased when placed into direct contact with porcine lymphocytes (**Figure 5**). Although this effect was relatively modest, the immunomodulatory activity of RPC populations warrants further investigation, as immunosuppressive activities would advocate for clinical use, while immunogenic activities would prohibit them.

Another consideration is that the isolation of RPCs in this study was from relatively healthy swine (**Table 1**). The use of normal, healthy, human donor kidneys for the isolation of RPCs might initially seem unnecessary given the growing number of patients on the kidney transplant list. However, RPCs may be isolated from diseased kidneys as well. For example, Gheisari et al. have shown that RPCs can be isolated and expanded from a mouse model of CKD (49), and that these cells are efficacious in a model of cisplatin-induced AKI. Moreover, a recent study also found that human primary cell cultures from both normal and CKD kidneys produced RPCs that had similar proliferative abilities, cell surface markers and phenotype (50). This brings forth the possibility that RPCs isolated from diseased kidneys from patients receiving transplants could be expanded and re-introduced in an autologous fashion to prolong the viability of the transplanted organ. The specific boundary conditions for this type of situation should be examined with preclinical animal models such as swine.

This study has several limitations worth mentioning. As mentioned earlier, this study only examines RPCs from one location within the kidney, and does not examine progenitor cells from, for example, the cortex. While the papillae represents one of the first (and thus extensively) characterized RPC population (19), it is now well recognized that many other such populations exist (24). Additionally, the degree of heterogeneity from subsequent passaging in, for example, differentiation capabilities was not quantified (i.e., Western blotting or RT-PCR). As such, we cannot rule out the possibility that this mixed cell population contains different progenitors with single lineage differentiation capacities. Also, experiments were limited by the relatively small amount of pig reagents and antibodies when compared to humans and rodents. Second, the phenotypic heterogeneity led to a low amount of harvestable cells (and thus biological replicates) for passage 6 which prevented analyses in the mixed lymphocyte reaction. Most importantly, these RPCs were not injected into any renal damage or disease model for examination of their therapeutic efficacy *in vivo*.

In conclusion, the current study shows that RPCs can be isolated from the renal papilla of porcine kidneys. Due to some of the limitations above, it is not clear whether this

represents a single, multipotent progenitor population, or several progenitor cell types with differentiation potential. Some heterogeneity is apparent in cell surface marker expression, and differentiation capabilities, however some of these properties are consistent with RPCs from other species. Regardless, the proliferative ability, and “stemness” properties of these cells are passage dependent, and might limit their expansion *ex vivo*. While swine will remain an important preclinical model for renal damage and transplantation, other populations of RPCs within the kidney should be investigated further.

DISCLOSURE

The opinions or assertions contained herein are the private views of the author and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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AUTHOR CONTRIBUTIONS

All authors participated in the interpretation of the studies and analysis of the data and also reviewed and approved the final version of the manuscript. MM, BG, and RM conducted the experiments; DB and MD were involved in the design of the studies. DB wrote the first draft of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effect of Intravenous Fluid Volumes on the Adrenal Glucocorticoid Response After Burn Injury in Swine

Belinda I. Gómez, PhD, Celestine He, Tony Chao, PhD, Michael A. Dubick, PhD, and David M. Burmeister, PhD

Severe thermal injury induces metabolic and physiological stress, prompting a disruption in the hypothalamic-pituitary-adrenal axis. The objective of this study was to evaluate potential confounding effects of Lactated Ringer's (LR) resuscitation on adrenal damage and cortisol production following burn. Anesthetized swine were instrumented with jugular catheters and sustained 40% TBSA burns from brass probes heated to 100°C. Animals recovered to consciousness and received IV fluid resuscitation with LR at two different volumes: 15 ml/kg/d (limited volume [LV], $n = 6$) or 2 ml/kg/%TBSA/d (modified Brooke [MB], $n = 6$). Nonburned animals (Sham) were both oral and IV fluid restricted (S-FR, $n = 4$) to induce stress. Computed tomography (CT) angiographies were performed at baseline (BL) and 48 hours postburn, while blood and urine samples were collected at BL, 6, 24, and 48 hours postburn, with euthanasia at 48 hours for adrenal harvesting. Urinary cortisol was elevated following burn/surgery in all animals and returned back to BL in S-FR (404 ± 48 pg/mg creatinine) but not MB (1332 ± 176 pg/mg creatinine; $P = .005$) or LV (1223 ± 335 pg/mg creatinine; $P = .07$) by 48 hours. Gene expression of cleavage enzymes (β -HSD, *CYP17*, *CYP11*, and *CYP21*) along the cortisol synthesis pathway showed minimal changes. Adrenal apoptosis (Terminal deoxynucleotidyl transferase dUTP nick-end labeling [TUNEL] staining) was greatest in the MB group ($P \leq .01$) when compared to S-FR, partly due to elevations in c-Jun N-terminal kinase. Adrenal hemorrhaging was also greatest in MB animals, with no differences in tissue volume or wet-to-dry ratio. However, tissue levels of cytokines IL-1 β , IL-10, and IL-12 were greatest in LV. Burn injury elevates urinary cortisol and compromises adrenal gland integrity, which is affected by IV fluid volume. (J Burn Care Res 2018;39:652–660)

Severe burns produce pathological changes that result in hemodynamic fluctuations, reduced organ perfusion, hypermetabolism, and a profound inflammatory response, all of which are known precursors to multiple organ dysfunction.¹ These systemic disturbances also initiate a stress response that lasts from the time of injury until well after wounds are healed. Acute fluid resuscitation is critical for survival and outcomes after burn injury, with the overall goal of maintaining adequate tissue perfusion. Currently, consensus guidelines set by the American Burn Association recommend lactated Ringer's solution (LR) at an initial rate of 2 to 4 ml/kg/% TBSA in the first 24 hours for intravenous (IV) fluid of burn injury. If not carefully titrated to urine output thereafter, excessive resuscitation volumes can cause edema/fluid overload, compartment syndromes, and acute respiratory distress syndrome^{2,3} further exacerbating stress. On the contrary, if patients are under-resuscitated, organ perfusion is not adequately maintained which leads to significant morbidity and mortality.

The hypothalamic-pituitary-adrenal (HPA) axis is responsible for the synthesis and secretion of glucocorticoids (ie, cortisol) whose metabolic effects are essential for adaptation to stress. The concentrations of plasma cortisol are elevated proportionally to the %TBSA burned.^{4–6} The body's response to burn stimuli coupled with emotional stress in conscious patients alters adrenocortical activity, further delaying healing. Chronic elevation of glucocorticoids is known to suppress the immune system and increase susceptibility to disease.⁷ The role of HPA axis in burn is characterized by dramatic alterations in cortisol levels, which may last months to years after patients survive the initial burn injury.^{8,9} Case reports, autopsy studies, and clinical investigations in patients with burns have demonstrated that damage and/or dysfunction of the adrenal glands (eg, hemorrhage, insufficiency) negatively impacts patient outcome.^{10–15} The incidence of adrenal hemorrhage in patients who succumbed to their burn injury was reported as 27.5% according to macroscopic observation during autopsy.¹⁰ Upon microscopic examination, most notable was the evidence of congested blood vessels within the adrenals of burn patients.¹¹

The prolonged elevation in cortisol secretion coupled with hemodynamic fluctuations challenge adrenal glands, and can be associated with adrenal hemorrhaging. Computed tomography (CT), ultrasound, magnetic resonance imaging, and autopsy findings are reported tools for diagnosing adrenal hemorrhage. Of the quarter of burn patients with adrenal hemorrhage, there is a higher prevalence in males.¹⁰ Although rare, acute adrenal insufficiency in patients with burns has been observed with rapid onset and sudden deterioration of the patient.¹⁵ Dysfunction of the HPA axis in response to other critical illnesses has recently been coined Critical Illness-Related Corticosteroid Insufficiency (CIRCI) in 2008. While

From the United States Army Institute of Surgical Research, JBSA Fort Sam Houston, Texas

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Address correspondence to David M. Burmeister PhD, United States Army Institute of Surgical Research, 3650 Chambers Pass, JBSA Fort Sam Houston, TX 78234. Email: David.m.burmeister3.civ@mail.mil

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adrenal insufficiency and its incidence in critically ill patients has been recognized for some time (first described in 1855)¹⁴ specific mechanistic research in burn injury is lacking.

Additionally, administration of hormones and hormone mimetics (eg, oxandrolone), following burn has generated recent interest and proven beneficial in some patients.¹⁶ This may render the physiological role of the HPA axis even more important. In rodent models, glucocorticoid receptor antagonist treatment has been shown to abolish burn-induced muscle proteolysis¹⁷ and expression of myostatin mRNA,¹⁸ as well as reduce apoptosis in the thymus and spleen.¹⁹ Since intact adrenal signaling is essential for patient outcome following burn injury, we aimed to investigate acute changes in burn-induced adrenal pathophysiology over the first 48 hours after injury. Furthermore, to identify if severity of damage is altered by resuscitation volumes, two different levels of IV LR was given at a low (15 ml/kg/d) and large (Modified Brooke Formula) doses. We hypothesized that IV LR fluids dose-dependently alter adrenal response after burn injury.

METHODS

Animals

Sixteen female Yorkshire swine weighing 41.4 ± 0.6 kg, free of parasites, and infection were included in this study. Animals had a minimum seven day acclimation period during which they were singly housed with ad libitum access to water, and fed a commercial laboratory pelleted diet formulated for pigs. Research was conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals, National Research Council. The facility's Institutional Animal Care and Use Committee approved all research conducted in this study. The facility where this research was conducted is fully accredited by AAALAC International.

Thermal Injury

Following the acclimation period, animals were fasted overnight and anesthetized with an intra-muscular injection of tiletamine-zolazepam (Telazol, 6 mg/kg), intubated, and placed on a ventilator with an initial tidal volume at 10 ml/kg, a peak inspiratory pressure at 20 cm H₂O, and respiration rate of 8 to 10 breaths/min. End tidal PCO₂ of 40 ± 5 mm Hg was maintained on the ventilator and 1 to 3% isoflurane. Creation of the burn wounds and postoperation animal care were performed as previously described.^{20,21} Briefly, hair was removed from the dorsum, flanks, and legs using clippers and razors with shaving cream. For the sampling of blood and administration of IV fluids, standard cut-down procedures were used to place left and right jugular vein catheters, which were anchored in place and tunneled subcutaneously to the back of the neck. All animals (including shams) were given a one-time (0.1–0.24 mg/kg) intramuscular injection of Buprenex-HCl Sustained Release (Veterinary Technologies/ZooPharm, Windsor, CO) for analgesia which the manufacturers have shown to be bioavailable in large animals for 72 hours. Large (9 × 15 cm) and small (5 × 5 cm) custom designed brass blocks equipped with a thermocouple were

maintained at $100 \pm 0.2^{\circ}\text{C}$ by a temperature controller. Heated probes were placed against the skin for 30 seconds to produce full thickness burn injuries as previously described,²² which was repeated until 40% of the TBSA was burned^{22,23} (Supplementary Figure 1). Burn wounds were covered with Ioban antimicrobial dressings (3M, St. Paul, MN) for the duration of the experiment and replaced if wounds were exposed. All animals were monitored constantly during the day through interaction and remote vivarium camera access to monitor health and behavior.

Animal Treatment Groups

During the experimental treatment, all animals had unlimited access to the dry pelleted pig diet, while only burned animals were given 15 ml/kg/d of drinking fluids. Animals were randomly assigned to one of three study groups. The first group was IV fluid resuscitation with LR at a limited volume (LV) of 15 ml/kg/d ($n = 6$). The second group received a larger IV volume of LR that was calculated according to the predicted standard of care modified brooke (MB) formula ($2 \text{ ml/kg/\%TBSA/d}$) ($n = 6$). Nonburned animals (Sham) underwent the same procedures except with room temperature blocks, but were deprived access to both oral and IV fluids (S-FR) to induce some level of stress. Animals recovered to full consciousness and were kept in a metabolic cage for separation of urine and feces, which also allowed for control of administering IV and oral fluids according to the treatment group. Resuscitation was performed through the indwelling jugular vein catheter via an infusion pump primed with sterile LR warmed to 37°C according to their body weight the morning of study. For the LV group, the entire volume was infused within 15 minutes, with the same volumes given on day 1 and day 2. For the MB group, on the first day half of the LR was administered in the first 8 hours and the other half in the next 16 according to standard care practice. For the MB group on the second day, while the total volume given was the same, the rate was set constant throughout the course of the day. If animals showed signs of distress (eg, vocalization, jumping, aggression) they were administered Midazolam (0.1–0.25 mg/kg) IM. Twenty-four and forty-eight hours following creation of the burn injuries, animals were mildly sedated with Telazol (6 mg/kg) to collect blood samples and record physiological parameters such as heart rate, respiratory rate, and rectal temperature. Heart rate and respiratory rate were measured manually by a veterinary technician by counting the number of heart beats and breaths for 15 seconds, and then multiplying by 4.

CT Angiography

At baseline (BL) and 48 hours, contrast-enhanced angiographies were performed under anesthesia. Animals were positioned prone, and 40 ml contrast agent (Isovue-370; Iopamidol 755 mg/ml; contains sodium 0.053 mg, organically bound iodine 370 mg/ml) was injected via an ear vein catheter and CT angiographies were initiated. The left adrenal was reconstructed for quantification of volume and Hounsfield units (HU) using VitreaAdvanced Version 6.7.4 (Vital Image Inc., Minnetonka, MN). After CT scanning,

euthanasia was performed with 10 ml of Fatal Plus (Vortech Pharmaceuticals, Dearborn, MI), and adrenal glands were either snap frozen in liquid nitrogen with storage at -80°C , or preserved in 10% neutral buffered formalin, with a ~ 1 g subsection saved for wet-to-dry weight analysis.

Histology

Adrenals were preserved in 10% neutral buffered formalin for a minimum of 48 hours, embedded in paraffin wax, and sectioned into $4\text{ }\mu\text{m}$ slices. Hematoxylin and Eosin (H&E) staining was performed according to the manufacturer's instructions (Sigma Life Science, St. Louis, MI). Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) and 4',6-diamidino-2-phenylindole (DAPI) stains were performed according to the manufacturer's protocol. Whole adrenal slices were imaged using an AxioScanZ1 slide scanner (Carl Zeiss, Thornwood, NY). Adrenal sections were put through automated quantification of colors with ImageJ software version 1.51d (Bethesda, MD). This software is equipped with a specific H&E color deconvolution tool that separates colors into channels. The pink/red channel containing red blood cells was set to a threshold of 90 and 255 allowing for an objective quantification of hemorrhaging throughout the entire cross-section of the adrenal. Three regions of interest within the adrenal to include: the glomerulosa, fasciculata/reticularis, and medulla were outlined using the region of interest tool. Within each region of interest within the adrenal cross-section the % surface area value of the pink/red channel was used as an indicator of red blood cell abundance (ie, hemorrhaging). For the analysis of TUNEL staining, four high-magnification images were taken from both the adrenal cortex and the adrenal medulla for each animal. Ensuing images were separated into red, green, and blue channels for the quantification of channel intensities, with apoptosis reported as the mean intensity of the green channel.

Blood and Urine Analysis

At BL and hours 6, 12, 24, and 48, urine samples were collected into 50 ml tubes, and blood samples were collected into K_2 EDTA containing tubes and centrifuged at $4300\times g$ for 10 minutes. Plasma was aliquoted, and stored at -80°C until analysis. Cortisol kit was purchased from Cayman (Ann Arbor, MI) and performed according to the manufacturers' protocol for plasma and urine. Blood samples were also collected into a lithium heparin containing tube and centrifuged at $4300\times g$. Serum glucose and cholesterol, as well as urine creatinine were analyzed on a Siemens Dimension Xp and Plus Clinical Chemistry System.

Real-time Polymerase Chain Reaction and Protein Quantification

RNA was isolated from snap-frozen adrenal glands using Trizol (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. RNA quantity was obtained using Nanodrop ND 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) at 260 nm and 280 nm. Genomic DNA removal and first-strand cDNA synthesis was performed according to the RT² First Strand Kit from (Qiagen, Hilden, Germany). Prepared cDNA samples were diluted in SYBR

(Bio-Rad Laboratories, Hercules, CA) with previously published primers²⁴⁻²⁶ and analyzed in duplicate using a i-Q5 real-time polymerase chain reaction detection system. Sequence specificity was completed using a melting curve with a 0.5°C temperature decrease from 55 to 95°C . Reference genes used to standardize variability include cyclophilin A (*Ppia*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). For protein cytokine analysis, a porcine-specific multiplex kit (EMD Millipore, Billerica, MA) was used according to the manufacturer's instructions.

Statistical Analysis

GraphPad Prism was used for graphical representation of data and statistical analysis. Data with repeated measures and histological analysis within the glomerulosa, fasciculata/reticularis, and medulla of the adrenal gland were analyzed using two-way analysis of variance method. Adrenal volume and perfusion were analyzed using a one-way analysis of variance. Protein levels, and wet-to-dry ratios were analyzed using unpaired *t* test. All data are presented as mean $\pm$ SEM.

RESULTS

Burn Injury Elevates Temperature

One animal from the S-FR group did not tolerate the metabolic cage (alertness to the point of excessive vocalization, jumping, etc.) and was therefore removed from the study. Table 1 shows changes in rectal temperature, heart rate, and respiratory rate at BL, 24, and 48 hours following burn injury. At 48 hours, temperature was elevated ($P = .005$) in LV ($39.7 \pm 0.3^{\circ}\text{C}$) and MB ($39.7 \pm 0.03^{\circ}\text{C}$) when compared with the S-FR animals ($38.5 \pm 0.2^{\circ}\text{C}$). Heart and respiratory rate were similar at BL and postinjury in all animals regardless of treatment.

Circulating glucose (a potential indicator of cortisol-induced hyperglycemia) was variable between timepoints due to feeding schedule. Collectively in all animals, plasma glucose (Table 1) was elevated by 24 hours ($180.94 \pm 10.73\text{ mg/dl}$; $P < .001$) and 48 hours ($189.00 \pm 31.85\text{ mg/dl}$; $P < .001$) when compared with BL levels ($70.67 \pm 6.58\text{ mg/dl}$). Importantly, all of these timepoints represent blood draws that occurred in the morning time after overnight fasting. Despite a trend for reduction toward BL levels in the MB group, this did not reach significant reduction.

Cortisol Is Elevated With Burn

Conversion of cholesterol to cortisol is regulated by expression of key steroidogenic enzymes in the adrenal gland (Figure 1A). Figure 1A depicts fold regulation of genes involved in cortisol synthesis relative to S-FR evaluated 48 hours following burn injury. A slight downregulation in cytochrome P450 family 11 subfamily A member 1 (*CYP11a1*), Cytochrome P450 Family 17 Subfamily A Member 1 (*CYP17a1*), β -Hydroxysteroid dehydrogenase (β -HSD), Cytochrome P450 Family 21 Subfamily A Member 2 (*CYP21a2*) was apparent after burn, but only *Cyp17a1* approached statistical significance ($P \leq .08$). It is important to note that transcriptional changes can happen on the order of hours, and 2 days may not be optimal to detect differences in this model.

Table 1. Temperature (°C), heart rate (beats/min), respiratory rate (breaths/min), and glucose (mg/dl) were collected at BL, 24 and 48 h after burn injury

Timepoint	Temperature			Heart Rate			Respiratory Rate			Glucose		
	S-FR	LV	MB	S-FR	LV	MB	S-FR	LV	MB	S-FR	LV	MB
BL	38.5 ± 0.2	38.6 ± 0.2	38.4 ± 0.3	148 ± 22	126 ± 12	135 ± 5	33 ± 3	46 ± 4	43 ± 3	77.3 ± 8.2	57.5 ± 7.9	77.2 ± 5.2
24 h	38.8 ± 0.1	39.3 ± 0.2	38.7 ± 0.2	136 ± 11	136 ± 15	120 ± 10	52 ± 11	43 ± 6	46 ± 10	192.3 ± 38.2	191.0* ± 25.3	159.5 ± 16.8
48 h	38.5 ± 0.2	39.7* ± 0.3	39.7* ± 0.3	128 ± 14	138 ± 14	141 ± 8	38 ± 8	41 ± 5	33 ± 1	196.0 ± 28.7	240.3* ± 59.8	130.7 ± 22.3

Values are presented as mean ± SEM and * indicates a significant ($P < .05$) difference from the BL value. S-FR ($n = 3$), LV ($n = 6$), MB ($n = 6$). BL, baseline; LV, limited volume; MB, modified brooke.

Cholesterol levels (Figure 1B) in all animals remained constant relative to BL levels until 48 hours when S-FR animals tended to display greater values ($P = .065$) relative to their BL values (92.33 ± 3.48 vs 66.33 ± 1.86 mg/dl, respectively) and were greater than MB animal values (58.67 ± 5.02 mg/dl; $P = .01$). Surgical preparations performed prior to burn (ie, animal cage transfer, sedation, surgical cut down, catheter line placement) proved stressful as BL plasma cortisol values in all animals were elevated (59.95 ± 8.87 ng/ml) and reduced at 48 hours in S-FR (20.54 ± 3.07 ng/ml), LV (21.46 ± 8.23 ng/ml), and MB (35.44 ± 6.87 ng/ml). Alternatively, urinary cortisol (Figure 1C) at BL was relatively low in all animals (85 ± 11 ng/mg creatinine), and therefore used for subsequent measurements. Six hours following the injury cortisol increased in all groups including SF-R (157 ± 19 ng/mg creatinine), LV (238 ± 44 ng/mg creatinine), and MB (211 ± 57 ng/mg creatinine) animals. At 24 hours in LV and MB animals (158 ± 41 and 139 ± 302 ng/mg creatinine, respectively) urinary cortisol remained elevated, whereas urinary cortisol levels in S-FR animals fell to near BL (64 ± 8 ng/mg creatinine). By 48 hours, levels slightly decreased in LV (122 ± 33 ng/mg creatinine) and MB (133 ± 17 ng/mg creatinine) and were statistically similar to their BL levels. However, cortisol levels remained greater in MB ($P = .0013$) and LV ($P = .03$) when compared with S-FR (40 ± 5 ng/ml).

CT Imaging of Adrenal Glands Did Not Detect Changes in Volume

At necropsy, no gross abnormalities were observed of the collected adrenal gland in any animals as they all were intact, of similar shape and size, and bright red in color (Figure 2A). To assess adrenal gland integrity, CT angiographies were taken at BL and 48 hours (Figure 2B). No significant differences were detected at BL between treatments for adrenal gland volume ($P = .97$; Figure 2C) and perfusion ($P = .35$; Figure 2D). Volume tended ($P = .12$) to be greater in MB animals when compared to S-FR animals. Hounsfield units as a measure of perfusion was reduced ($P = .009$) to (94.7 ± 2.76 HU) 48 hours following BL (106.9 ± 3.98 HU) in all burned animals. Perfusion at 48 hours was greatest in S-FR (108.5 ± 6.7 HU; $P = .03$) when compared to LV (95.1 ± 3.6 HU) and MB (88.18 ± 3.2 HU; Figure 2D). Despite these observations, wet-to-dry adrenal gland weights were similar among groups (Figure 2E).

Histopathology Showing Adrenal Hemorrhage and Apoptosis

Representative images for adrenal sections stained for H&E are shown in Figure 3, which illustrate varying levels of hemorrhage in all animals and amongst different locations within the adrenal (Figure 3A–F). Severity of hemorrhage was greatest in the glomerulosa ($P = .004$) of MB animals when compared with S-FR or LV ($P = .08$; Figure 3G). While similar trends were seen in other locations, no significant differences were detected within the fasciculata/reticularis, or the medulla. To quantify the level of apoptosis-mediated cellular death within the cortex and medulla of the adrenal gland, fixed sections were stained with TUNEL and DAPI.²⁷ Intensity of TUNEL stain on adrenal cortex sections was greater in the

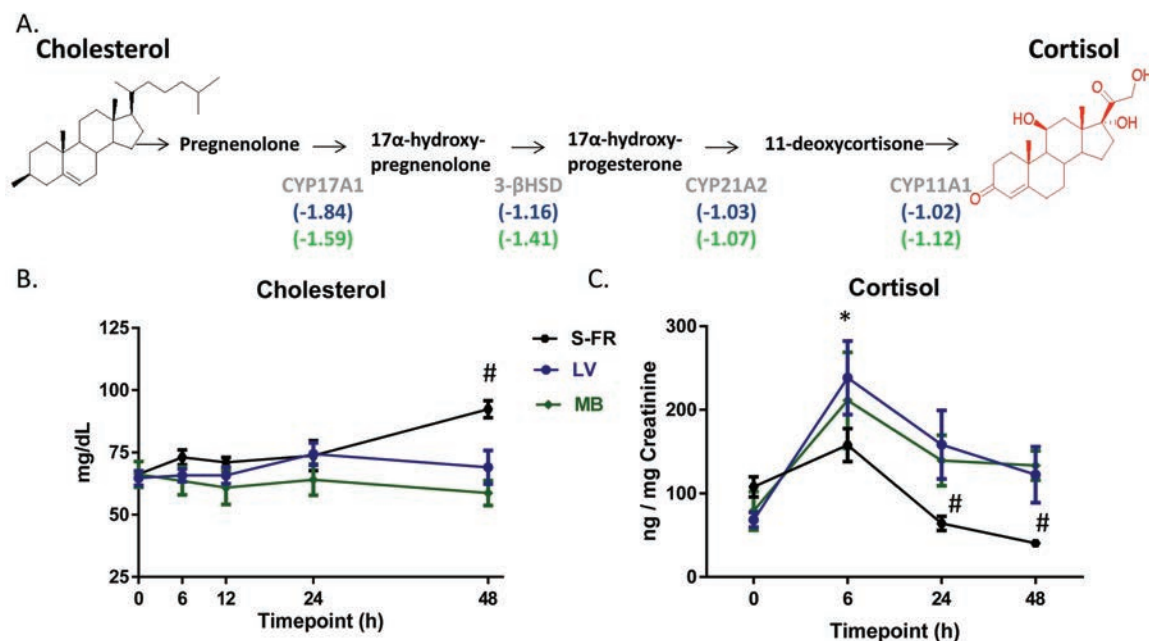


Figure 1. Diagram of cholesterol to cortisol synthesis and the steroidogenic enzymes (gray) required for conversion (A). Fold Regulation (blue LV and green MB) of steroidogenic enzymes cytochrome P450 family 11 subfamily A member 1 (*CYP11a1*), Cytochrome P450 Family 17 Subfamily A Member 1 (*CYP17a1*), 3 β -Hydroxysteroid dehydrogenase (3 β -HSD), Cytochrome P450 Family 21 Subfamily A Member 2 (*CYP21a2*). Plasma cholesterol (B) and urinary cortisol (C) are quantified at baseline (BL), 6, 12, 24, and 48 hours following burn injury. Values are presented as mean $\pm$ SEM and * indicates a significant ($P < .05$) difference from the BL value and # indicates a difference ($P < .05$) between treatment(s) at the indicated timepoint. S-FR ($n = 3$), LV ($n = 6$), MB ($n = 6$). LV, limited volume; MB, modified brooke.

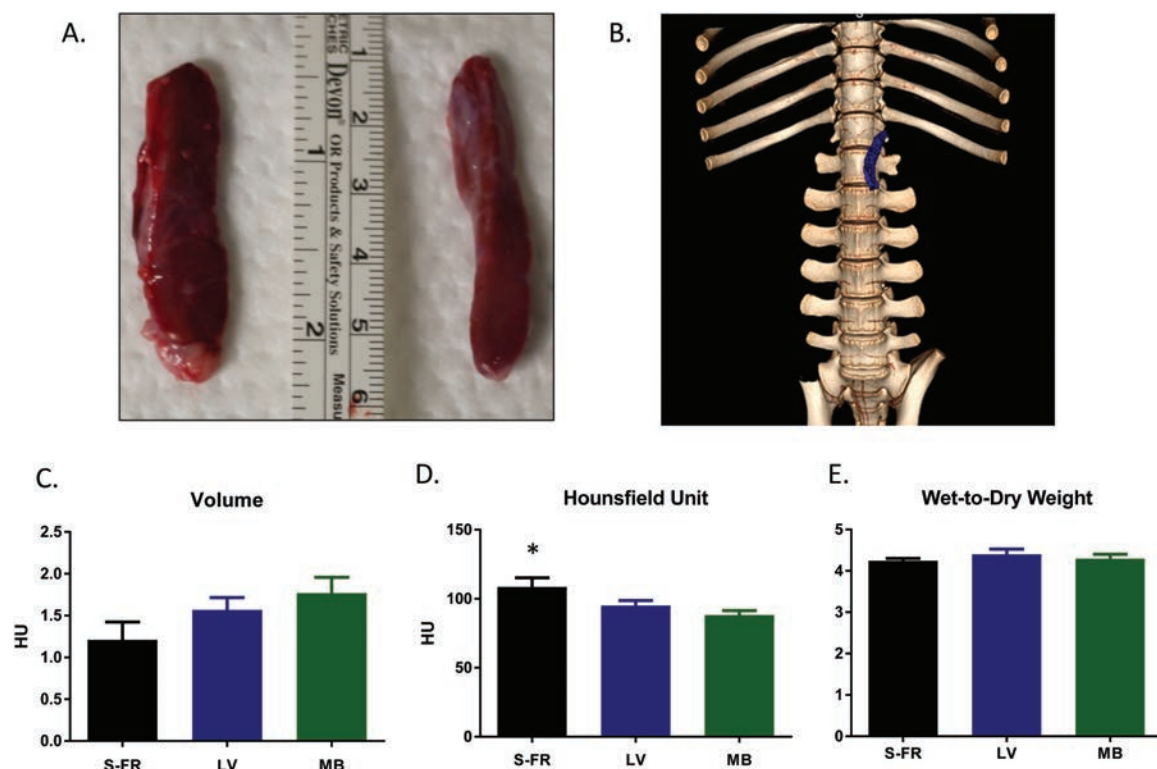


Figure 2. (A) Adrenal glands isolated immediately after euthanasia demonstrated no gross abnormalities between animals (representative picture shown is from MB group). (B) Computed tomography (CT) scanning was performed pre-injury and immediately prior to euthanasia (termination of experiment 48 hours; representative picture shown is from MB group). (C) Adrenal volume and Hounsfield units (D) were quantified at 48 hours. (E) Wet-to-dry values. Values are presented as mean $\pm$ SEM and * indicates a significant ($P < .05$) treatment difference.

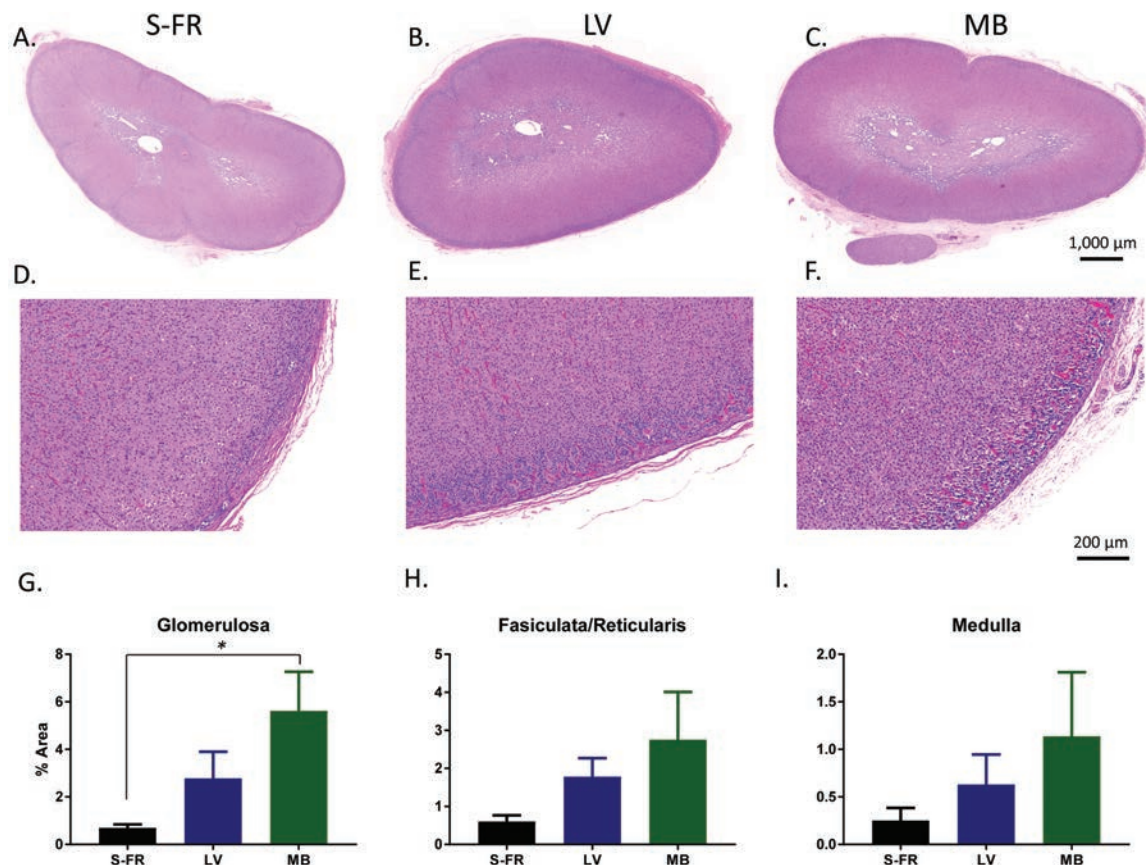


Figure 3. Representative hematoxylin and eosin (H&E) staining of the adrenal in S-FR (A and D), LV (B and E), and MB (C and F) animals. Images demonstrate hemorrhaging which are graphically represented as mean $\pm$ SEM of % surface area in the glomerulosa (G), fasciculata/reticularis (H), and medulla (I). S-FR ($n = 3$), LV ($n = 4$), MB ($n = 4$). Scale bars represent 1 mm for A–C, and 200 μ m for D–F. An * indicates a significant ($P < .05$) treatment difference. LV, limited volume; MB, modified brooke.

MB group ($P < .01$) and LV group ($P = .14$) when compared to S-FR (Figure 4C). Within the medulla, there was also detectable TUNEL staining in all animals, with a tendency (Figure 4D) for greater expression in the MB group ($P = .14$). The apoptosis-mediating molecule c-Jun N-terminal kinase (JNK) (Figure 4E) was quantified in total adrenal homogenates and levels were significantly greater in MB ($P = .03$), and moderately greater in LV ($P = .10$) animals when compared with SF-R.

Cytokines Are Elevated With Low Volume IV Resuscitation

Proinflammatory and anti-inflammatory cytokine levels in adrenal lysates were quantified (Figure 5). Within the tissue, levels of anti-inflammatory cytokines IL-10 ($P = .03$) and IL-1ra ($P < .05$) were greater in LV when compared with MB animals. IL-4 tended to be greater in S-FR animals when compared to MB ($P = .08$). However, proinflammatory cytokines IL-12 and IL-1 β were also greatest in LV animals when compared to S-FR ($P = .04$ and $P = .13$, respectively) and MB ($P = .02$ and $P = .13$, respectively) animals. Alternatively, levels of IL-6 tended to be greater in MB ($P = .02$) and LV ($P = .10$) animals when compared with S-FR.

DISCUSSION

Patients with burns experience fluctuations in plasma cortisol that are proportional to the size of the burn.⁴ Typically this results in greater circulating cortisol concentrations and increased cortisol production lasting several days after burn.^{4,28} With all of the pathological stressors that take place after burn injury, the role of the adrenal gland is crucial for patient outcome. For example, Curling's ulcer, an acute ulceration of the stomach or duodenum in some patients with burns, may be ultimately brought on by elevated stress and cortisol.^{29,30} Although changes in cortisol are a normal adaptation mechanism to stress and injury, if left unregulated for a period of time, it can lead to detrimental outcomes. Many researchers have demonstrated cortisol fluctuations following burn injury, and while mechanistic examination of the adrenal response following burn is possible with animal models, this remains largely unstudied. The current study utilizes a porcine 40% TBSA burn model to report that volumes of IV resuscitation with LR can affect the response of the adrenal following burn injury. Specifically, although greater volumes of fluids exacerbated hemorrhaging and apoptosis postburn within the adrenal gland, this did not cause an increased inflammatory profile.

Other salient findings presented herein demonstrate that urinary cortisol is increased after burn injury with no major effect

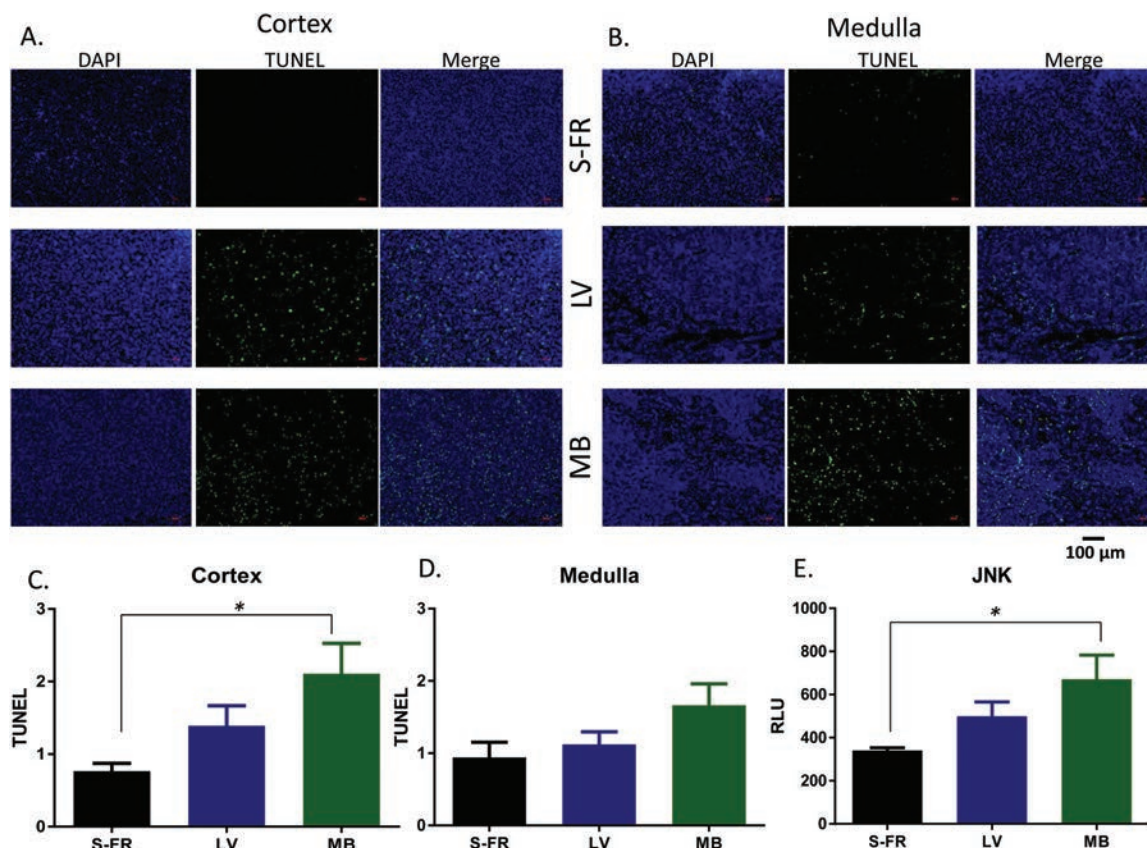


Figure 4. Representative adrenal TUNEL- and DAPI-stained adrenal cortex (A) and medulla (B) sections showing staining intensity and thus apoptosis between S-FR, LV, and MOB treatments. (C) Quantification of color density reveals significantly greater apoptosis in the cortex and medulla (D) of MB-treated swine. Total JNK protein expression in adrenals was higher in the MB when compared to the S-FR and LV groups (E). Values are presented as mean $\pm$ SEM. S-FR ($n = 3$), LV ($n = 4$), MB ($n = 4$) and * indicates a significant ($P < .05$) treatment difference. Scale bars for all images represent 100 μ m. JNK, c-Jun N-terminal kinase; LV, limited volume; MB, modified brooke; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick-end labeling.

of IV fluid volume, indicating this may represent a noninvasive marker of the transition from the “ebb” (shock) phase to the “flow” (protein and fat turnover for energy maintenance) phase of burn pathogenesis.³¹ While the temporal aspect of urinary cortisol concentrations needs further delineation, this type of information could inform treatments strategy such as timing of surgical intervention. In this study burned pigs had greater cortisol levels than S-FR, but they recovered to near BL levels by 48 hours. Interestingly, control animals that were not burned (S-FR) displayed more stressed behavior and therefore required midazolam. The reason for this behavior is likely from S-FR animals not being thermally injured and therefore are more aware of the metabolic cage environment (ie, the burn injury itself caused mild sedation and lethargy). Despite this, S-FR animals’ cortisol levels were not as consistently elevated as the burned animals.

Patients with burns >20% TBSA have a 40% reduced cholesterol that correlates to greater infection rates and length of stay, as well as a prolonged reduction in cortisol.³² Total cholesterol was measured to ascertain whether burn injury similarly depletes levels in this swine model, and if this is affected by changes in cortisol production due to the resuscitation regimen. Interestingly, we found no such relationship herein, and key steroidogenic enzymes responsible for the

production of cortisol were actually lower than sham animals, albeit nonsignificantly. As mentioned earlier, the expression of these enzymes may have risen sharply after burn injury, and had already decreased to BL values by 48 hours. Chronic stress has been reported to increase adrenal gland weight in rats; therefore, a CT scan at BL and 48 hours was performed to detect changes in adrenal gland volume and perfusion.³³ While minor changes in adrenal volume were detected, significant changes may be realized with an increase in animal number. On the contrary, burn injury reduced perfusion of the adrenal (HU; Figure 2) as S-FR animals displayed similar levels to BL that were significantly higher than both groups at 48 hours.

Postmortem evaluation of adrenal glands provided more insight into the degree of burn-induced damage. Hemorrhage was not macroscopically observed in the adrenals during organ harvest at necropsy, as has been reported in some patients with burns.^{10,11} However, histological observation demonstrated hemorrhaging and cellular death postburn. Furthermore, animals receiving larger fluid volumes displayed greater hemorrhaging and apoptosis in the adrenals when compared to S-FR animals. Moreover, adrenal hemorrhaging in the glomerulosa and TUNEL expression measured histologically were positively correlated with each other ($r = .77$, $P = .006$). While the

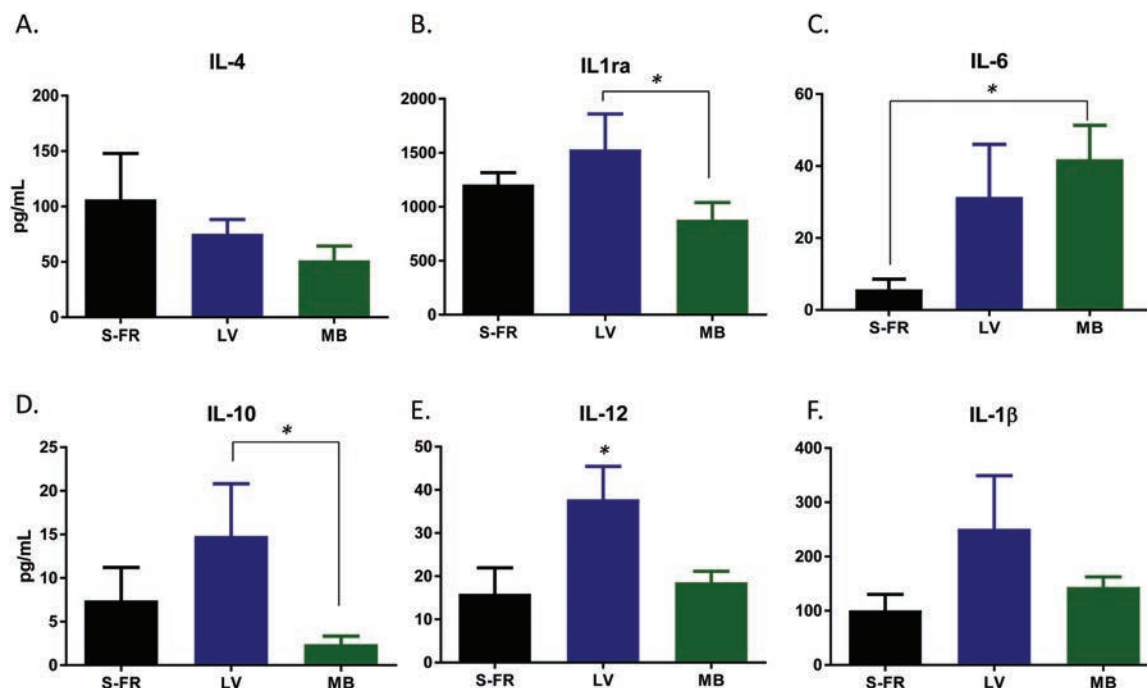


Figure 5. Cytokines IL-4 (A), IL1ra (B), IL-6 (C), IL-10 (D), IL-12 (E), and IL-1β (F) were quantified in adrenal tissue and values are presented as mean ± SEM of the observed concentration and * indicates a significant ($P < .05$) treatment difference. S-FR ($n = 3$), LV ($n = 4$), MB ($n = 5$). LV, limited volume; MB, modified brooke.

implications of this relationship is unclear, JNK proved to be a key signaling molecule mediating apoptosis in this model, as expression of JNK also positively correlated to TUNEL staining within both the cortex ($R = .71$, $P = .01$) and medulla ($R = .62$, $P = .04$). The drawbacks or benefits of apoptosis within the adrenal postburn injury warrants further investigation, as many different molecular targets exist that could be leveraged for therapeutic purposes.

Given the importance of IV fluids in burn injury, the above TUNEL stain finding may seem counter-intuitive. Indeed, limiting IV fluids in the burn patient can lead to deleterious consequences as proven by improved outcomes using large volumes in the last several decades. As such, the increased apoptosis may represent a protective mechanism in which programmed death of compromised cells potentiate recovery of the HPA axis. We have recently shown that limiting volumes of enteral fluids in this model exacerbates AKI.³⁴ Moreover, a recent study suggested that limited volumes of IV fluids (although still within the range of 2–4 ml/kg/%TBSA) may predispose patients to AKI.³⁵ In our study, the low volume group did show a low urine output and elevated creatinine suggesting that volumes of IV fluids below the standard of care are not optimal. While limited IV fluids may be a problem in austere environments and mass casualty scenarios, the nationwide shortage of IV fluids due to the recent hurricanes in Puerto Rico may also limit fluid administration. In this light the use of alternative IV fluids or oral resuscitation for the treatment of burn treatment warrants further investigation.

While previous investigators identified that cholesterol was inversely correlated to plasma IL-6,³² cytokine levels in adrenal tissue after burn injury has not been reported to date. In our model, protein levels of IL-6 in adrenal tissue

were greatest in the MB group when compared to the other groups. These results align with several others demonstrating IL-6 levels correlate with burn injury and severity.^{36,37} Additionally, adrenal IL1ra and IL-10 levels were greatest in the LV relative to the MB group. While these cytokines are also implicated in burns,^{38,39} this data would seem to indicate that a conservative fluid regimen increases levels of anti-inflammatory cytokines within adrenal tissue. However, this phenomenon was nonspecific, as levels of proinflammatory cytokine IL-12 was also higher with limited fluids. In this regard, greater volumes of IV fluids given in our MB group reduced levels of most cytokines, with the exception of IL-6. However, no differences were detected in wet-to-dry ratios, thus eliminating the possibility of simple dilution of these proteins. Contrary to our hypothesis, the apoptosis and hemorrhaging mentioned above did not result in a locally exacerbated inflammation.

There are limitations of this study worth mentioning. Swine are highly intelligent animals and cognitive stress associated with abrupt changes in their normal day to day activities (eg, surgery preparation)⁴⁰ prevented normal, unstressed plasma cortisol measurements. Further investigation on cortisol levels could utilize urinary or salivary measurements as a nonstress-inducing method of obtaining measurements. Although this study was not intended to analyze animal behavior after burn, nonburned animals displayed behavior of being more stressed from placement into the metabolic cage, which necessitated the exclusion of one animal from the S-FR group. This negatively affected detection of significance levels, and there are several examples of data presented herein that approached statistical significance. Along the same lines, the greater volume of fluids administered in this study (ie,

MB) represents a moderate level of IV fluids that is at the lower end of the standard of care spectrum. IV fluid volumes according to the Parkland formula (4 ml/kg/%TBSA/d, double that given in this study) would have likely exacerbated the volume-associated findings in hemorrhaging and apoptosis, we chose to study more limited volumes for application to resource poor setting (eg, prolonged field care, wilderness medicine, mass casualty events). Other limitations are commonly associated with swine, and include the lack of appropriate molecular tools (eg, antibodies/primers) and high cost precluding the analysis of tissue protein and gene expression over time. Nevertheless, we believe that our model can offer insights into the early stress response, the mechanisms associated with the cortisol response, and the role of the adrenal gland postburn injury.

CONCLUSION

Patients with burns experience fluctuations in cortisol production that are normal to help cope with the degree of injury. This study demonstrates in a controlled animal study that urinary cortisol levels may provide a noninvasive diagnostic tool delineating the transition from ebb to flow phases of burn injury. Moreover, cellular death (particularly in the adrenal medulla) is exacerbated with greater volumes of resuscitation fluid which is also associated with adrenal hemorrhaging. This does not, however, lead to inflammation as both pro- and anti-inflammatory cytokines in adrenal tissue were nonspecifically regulated. The findings in this study highlight the acute effects of burn on the adrenal gland and suggest further mechanistic studies are warranted.

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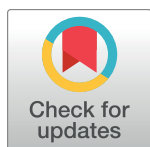
RESEARCH ARTICLE

Enteral resuscitation with oral rehydration solution to reduce acute kidney injury in burn victims: Evidence from a porcine model

Belinda I. Gómez¹, Matthew K. McIntyre¹, Jennifer M. Gurney^{1,2}, Kevin K. Chung^{2,3}, Leopoldo C. Cancio^{1,2}, Michael A. Dubick¹, David M. Burmeister^{1*}

1 United States Army Institute of Surgical Research, Fort Sam Houston, TX, United States of America, **2** Brooke Army Medical Center, Fort Sam Houston, TX, United States of America, **3** Uniformed Services University of the Health Sciences, Bethesda, MD, United States of America

* David.m.burmeister3.civ@mail.mil



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Abstract

Intravenous (IV) resuscitation of burn patients has greatly improved outcomes and become a cornerstone of modern burn care. However, the heavy fluids and vascular access required may not be feasible in austere environments, mass casualty, or delayed transport scenarios. Enteral resuscitation has been proposed for these situations; we sought to examine the effectiveness of this strategy on improving burn-induced kidney injury. Anesthetized Yorkshire swine sustaining 40% TBSA full-thickness contact burns were randomized to three groups ($n = 6/\text{group}$): fluid deprivation, ad libitum water access, or 70 mL/kg/d Oral Rehydration Salt solution (ORS). Urine and blood were collected at baseline (BL), 6, 12, 24, 32, and 48h post-burn, at which point tissue was harvested and CT angiography performed. Although fluid consumption by ad libitum and ORS groups were matched ($132 \pm 54 \text{ mL/kg}$ versus $120 \pm 24 \text{ mL/kg}$, respectively), ORS intake increased urine output compared with water and no water ($47.3 \pm 9.0 \text{ mL/kg}$ versus $16.1 \pm 2.5 \text{ mL/kg}$, and $24.5 \pm 1.7 \text{ mL/kg}$ respectively). Plasma creatinine peaked 6h following burn ($1.67 \pm 0.07 \text{ mg/dL}$) in all animals, but at 48h was comparable to BL in animals receiving water ($1.23 \pm 0.06 \text{ mg/dL}$) and ORS ($1.30 \pm 0.09 \text{ mg/dL}$), but not fluid deprived animals ($1.56 \pm 0.05 \text{ mg/dL}$) ($P < 0.05$). Circulating levels of blood urea nitrogen steadily increased, but also decreased by 48h in animals receiving enteral fluids ($P < 0.05$). Water deprivation reduced renal artery diameter ($-1.4 \pm 0.17 \text{ mm}$), whereas resuscitation with water ($-0.44 \pm 0.14 \text{ mm}$) or ORS maintained it ($-0.63 \pm 0.20 \text{ mm}$; $P < 0.02$). Circulating cytokines IL-1 β , IL-6, IFN γ , and GM-CSF were moderately elevated in the fluid-deprived group. Taken together, the data suggest that enteral resuscitation with ORS rescues kidney function following burn injury. Incorporating enteral fluids may improve outcomes in resource-poor environments and possibly reduce IV fluid requirements to prevent co-morbidities associated with over-resuscitation. Studies into different volumes/types of enteral fluids are warranted. While ORS has saved many lives in cholera-associated dehydration, it should be investigated further for use in burn patients.

Introduction

Worldwide, one of every ten deaths is a result of trauma which is also the number one cause of mortality among individuals under 40 years of age; burns are the fourth most common type of trauma [1]. In the year 2015, over 180,000 deaths were a result of fire or other hot substances [2]. Severe burn injury elicits a complex physiologic response resulting in diminished plasma volume, hypermetabolism, and a profound inflammatory response which often results in multiple organ dysfunction (MOD). The kidneys are frequently affected in MOD and there is a high incidence of acute kidney injury (AKI) that occurs in burn patients [3, 4]. AKI is also independently associated with increased mortality in thermal injury [5±7]. Associated problems of AKI include retention of blood urea nitrogen (BUN), volume overload, reduced antioxidant status, altered immunologic responses, and mitochondrial damage within the kidney [8].

As a surrogate for kidney function, urine output is the main indicator that guides resuscitation with intravenous (IV) fluids in burn patients. Indeed, the realization that IV resuscitation and maintenance of intravascular volume decreases comorbidities such as AKI and maintains end-organ perfusion has revolutionized burn care and improved patient outcomes. Initial fluid volume infusion rates in burn patients are commonly given in the range of 2±4 ml/kg/%TBSA, representing the range of the modified Brooke (2 ml/kg/%TBSA) and Parkland (4 ml/kg/%TBSA) formulas. However, fluid type and volume administered have yet to be standardized, leading to large variation in resuscitation protocols [9]. While IV fluid resuscitation remains the standard treatment for burn patients, the efficacy of oral rehydration therapy has been proposed for decades [10±12]. Past clinical trials and animal experiments utilized various formulations of simple electrolyte solutions and found them effective for the treatment of burn injury [13]. In disaster or resource-limited situations, enteral fluids may be the only option due to a lack of IV fluids or an inability to gain vascular access.

The Oral Rehydration Salt solution (ORS) according to the World Health Organization (WHO) is a simple formula that contains glucose, sodium chloride, potassium chloride, and trisodium citrate. ORS has been successfully used for decades to save millions of lives in third world countries from dehydration secondary to severe diarrhea in conditions such as cholera [14]. This suggests the feasibility of rapidly mobilizing these simple treatments in the wake of large-scale mass casualty incidents. Additionally, the relative ease of ORS implementation (e.g., drinking, or through a nasogastric tube) may aid in preserving organ function in delayed transport scenarios such as prolonged field care or wilderness medicine. An animal study of 40%TBSA burns in swine demonstrated that roughly 93% of ORS was absorbed, leading to greater urine output than with IV fluids [15]. A clinical study of 20 children with 10±20% TBSA burns found similar levels of urine output comparing enteral and IV fluids [16]. More recently, a randomized clinical trial evaluated enteral resuscitation versus IV fluid in adults with >15% TBSA burns and demonstrated greater urine output on day 3 post-burn in patients receiving enteral fluids [17]. These studies suggest ORS is safe and effective for burn injury, which not only may prove life-saving in the austere environments mentioned above, but also may reduce IV fluid requirements when the patient has reached definitive clinical care.

Despite these studies advocating for the feasibility and efficacy of ORS, the potential for enteral fluids to ameliorate burn-related comorbidities remains largely unstudied. As an initial step to demonstrate the efficacy and feasibility of ORS, we used a large animal model of moderate burn injury to assess the effects of ORS at reducing AKI in a controlled environment. We utilized a 40% TBSA burn conscious swine model. We hypothesized that ORS is reno-protective and is superior to water alone at maintaining kidney function and perfusion.

Materials and methods

Animals

The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee at United States Army Institute of Surgical Research (*Protocol number A16-011*). This study has been conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals. Approval was received prior to research and all efforts were made to minimize animal suffering. A completed ARRIVE guidelines checklist is provided as [S1 Fig](#).

Eighteen pre-pubertal female Yorkshire swine weighing 40.2 ± 2.1 kg, free of parasites and infection, were included in this study. Upon arrival to our Institute, animals had a minimum seven-day acclimation period, during which they were singly housed, with ad libitum access to water, and fed a commercial laboratory porcine formulated pelleted diet. Animals were randomly allocated to one of three treatments following thermal injury: fluid deprived ($n = 6$), ad libitum water access ($n = 6$), or 70 mL/kg/d of ORS (ORS; $n = 6$) for 48h. A gravity-fed spigot was customized using a carboy attached to a lixit via long flexible tubing and attached to a drip bowl on the metabolic cage. Fluid intake was carefully monitored by measuring fluid waste caught in the drip bowl that leaked from the spigot or the animal's mouth as she drank. For the animals receiving ORS, the amount of waste was replaced with fresh ORS to ensure the animal received the entire volume allotted. During the experimental treatment animals had unlimited access to the dry pelleted diet and special dry cookie treats until fasting before anesthetic events. After euthanasia with 10 mL of Fatal Plus (Vortech Pharmaceuticals, Dearborn, MI) at 48 h, kidneys were harvested and weighed and sections were preserved in 10% neutral buffered formalin until further processing. Additionally, 2 ± 3 grams of the kidney was blotted dry and weighed before and after drying in a 60°C oven for calculating wet-to-dry ratio.

Thermal injury

Creation of the burn wounds and post-operative animal care were performed as previously described [18]. Briefly, animals were anesthetized with an IM injection of tiletamine-zolazepam (Telazol, 6 mg/kg), intubated, and placed on a ventilator with an initial tidal volume at 10 mL/kg, a peak inspiratory pressure of 20 cm H₂O, and respiratory rate of 8 to 10 breaths/min. The ventilator was adjusted to achieve an end-tidal PCO₂ of 40 ± 5 mm Hg. Animals were maintained on 1% to 3% isoflurane, balance O₂ anesthesia. Hair was removed from the dorsum, flanks, and legs using clippers and razors with shaving cream. For the sampling of blood, standard cut-down procedures were used to place left and right external jugular vein catheters that were anchored in place and tunneled subcutaneously to the back of the neck. Large (9x15 cm) and small (5x5 cm) custom-designed brass blocks equipped with a thermocouple were maintained at 100 ± 0.2 °C by a temperature controller. Heated probes were placed against the skin for 30 s to produce full-thickness burn injuries as previously described [19]. This procedure was repeated until 40% of the TBSA was burned [20]. Animals did not receive intravenous resuscitation fluids throughout the study.

Animal care

All animals were given a one-time intramuscular injection of 0.1 ± 0.24 mg/kg Buprenex-HCl Sustained Release (Veterinary Technologies/ZooPharm, Windsor, CO), which provides analgesia for up to 72 hours, immediately prior to the creation of the burn wounds. Burn wounds were covered with Ioban antimicrobial dressings (3M, St. Paul, MN) for the duration of the experiment, which were replaced if wounds were exposed. Animals recovered and were kept

in a metabolic cage (dimensions 41' L x 16' W x 44' H) for collection of urine and monitoring of their enteral fluid intake. Feed was given once animals were awake and standing independently. Approximately 24 and 48 h following burn injury, animals were sedated with Telazol (6 mg/kg) to collect blood samples and monitor physiological parameters (heart rate, respiratory rate, and rectal temperature). During research period, individual animals were monitored at a minimum hourly (during normal work hours) by the veterinary technician staff, veterinarians, research staff, or husbandry staff for signs of distress. This was routinely done in a hands on fashion daily for all animals (S2 Fig), as well as documented twice a day on pain and distress sheets (S3 Fig) after injury. Additionally, monitoring was continuously done remotely via animal room camera access. If animals showed signs of distress (e.g., vocalization, jumping) they were administered midazolam (0.1 ± 0.25 mg/kg) IM for light sedation. In these scenarios, a sedative over additional analgesia was chosen because the root cause of distress was the animals' environment (i.e., the metabolic cage) and not pain per se. This was determined due to previous experience with a lack of distress when returned to the home cage [21], and an initial attempt at sham controls exhibiting increased aversion to the metabolic cage.

Computed tomography (CT) angiography

At baseline and 48 h, renal perfusion, volume, and renal artery diameter were assessed with contrast-enhanced angiography. Animals were anesthetized as described earlier, positioned prone, and 40 mL of contrast agent (Isovue-370; Iopamidol 755 mg/mL; contains sodium 0.053mg and organically bound iodine 370 mg/mL) was injected via ear vein catheter and CT angiographies were performed. Renal artery diameter, kidney volume and perfusion were quantified using Vitrea Advanced Version 6.7.4 (Vital Image Inc., Minnetonka, MN). Both right and left kidneys were selected using 2D slices to reconstruct the entire kidney and the measurement tool gave volume and Hounsfield units of the whole organ. For all parameters measured, changes from baseline to 48 h post burn were calculated for individual animals.

Histology

Upon euthanasia at 48 h, kidney samples were immediately preserved in 10% neutral buffered formalin, embedded in paraffin wax, and sectioned into 4- μ m slices. Periodic Acid Schiff (PAS) staining was performed according to the manufacturer's instructions (Sigma Life Science, St. Louis, MI) and tubular degeneration was scored on a scale of 0–5 for both distribution (0- none, 1- scattered, 2- <10%, 3-10–25%, 4-25–50%, and 5- >50%) and severity (0- none, 5- severe) by a blinded histopathologist. The scores were added together, leading to a score of 10 being the worst possible. Whole kidney slices were imaged using an AxioScanZ1 slide scanner (Carl Zeiss, Thornwood, NY). Images of entire sections were put through automated quantification of colors with ImageJ software version 1.51d (Bethesda, MD). The PAS color deconvolution tool was used to separate the pink and blue channels. Software-acquired measurements for each channel included the mean intensity and density.

Blood and urine analysis

Urine samples were collected into 50-mL tubes and blood samples were collected into K₂ EDTA-containing tubes and centrifuged at 4,300 x g for 10 min, aliquoted, and stored at -80°C until analysis. If there was no urine output overnight, a foley catheter was inserted while the animal was under anesthesia at 24 and 48 h as described above (6mg/kg Telazol IM), and 10mL of urine was aspirated for urinalysis. Superoxide dismutase (SOD) kit was purchased from Cayman (Ann Arbor, MI) and performed according to the manufacturers' protocol for plasma. Free hemin was quantified in the plasma using a commercially available colorimetric

assay kit (BioVision, Milpitas, CA) according to the product inset directions. For cytokine analysis, a porcine-specific multiplex kit (EMD Millipore, Billerica, MA) was used according to the manufacturer's instructions. Blood samples were also collected into a lithium-heparin-containing tube and centrifuged at 4,300 x g.

Serum and urine biochemical values were analyzed on a Siemens Dimension Xp and Plus Clinical Chemistry System. For complete blood count, blood was collected into K₂ EDTA containing tubes and analyzed with the Abbott Cell-Dyn 3700 system. For venous blood gas analysis, 1 mL of blood was collected and one drop was loaded into an iSTAT CG4+ cartridge. The cartridge was read using the iSTAT Portable Clinical Analyzer (Abbott Point of Care, Princeton, NJ). Finally, creatinine clearance (i.e., glomerular filtration rate, GFR) was calculated at each timepoint by the following equation: $((\text{Creatinine}_{\text{urine}} \times \text{Volume}_{\text{urine}}) / (\text{Creatinine}_{\text{plasma}} \times \text{Time}_{\text{min}}))$. Values across time for each animal were averaged to represent total creatinine clearance.

Statistical analysis

Statistical analysis was performed using JMP® (SAS institute, Inc, Cary, NC). Data with repeated measures were analyzed using 2-way analysis of variance method (ANOVA) followed by Tukey's or t-test for multiple comparisons. For analysis of histology, protein, fluid intake, urine output, GFR, kidney volume, artery diameter, and wet-dry ratios a 1-way ANOVA and Tukey's multiple comparison tests were performed. For these analyses, non-parametric testing was utilized where appropriate (e.g., histological analysis and GFR) or when Brown-Forsythe testing revealed that the variances amongst groups were different. All data are presented as mean ± standard error of the mean (SEM) using GraphPad Prism, which was also used to run linear regression analysis. Significance was set at $P < 0.05$.

Results

Burn injury alters physiological parameters

All animals recovered in a metabolic cage. They displayed an appetite the first day of the study but not on the second. No animals died prior to scheduled euthanasia. Seven animals (39%) required midazolam for mild sedation, but maintained full consciousness in the metabolic cage. By h 48 all animals displayed elevated body temperature (BL: 38.21 ± 0.03 vs 48 h: $40.06 \pm 0.09^\circ\text{C}$), respiratory rate (BL: 34 ± 5 vs. 48 h: 43 ± 3 breaths/minute), but not heart rate (BL: 138 ± 3 vs. 48 h: 138 ± 4 beats/minute). Additionally, Table 1 provides physiological parameters demonstrating severity of illness but no significant effect of treatment. Burn injury increased circulating white blood cell count by 6 h which remained elevated throughout the duration of the study ($P < 0.05$ vs baseline). All animals displayed alkalemia by 6 h following burn injury that returned to normal values by 24 h. Venous blood base excess in the extracellular fluid compartment (BE_{ecf}) in the fluid-deprived and the ORS group was elevated by 6 h ($P < 0.05$) relative to BL values.

ORS reduces burn-induced acute kidney injury

The total fluid volume consumed over the 48 h by the ad libitum water and 70 ml/kg/d ORS was not significantly different ($5,488 \pm 947$ mL (132 ± 54 mL/kg) versus $4,812 \pm 373$ mL (120 ± 24 mL/kg), respectively; Fig 1A). However, ORS consumption led to a significantly greater total urine output, which was nearly tripled when compared with water and doubled when compared with fluid-deprived groups ($1,894 \pm 361$ mL versus 664 ± 113 mL, and 902 ± 47 mL respectively; $P < 0.05$). Additionally, urine output normalized for weight is still greatest with ORS (47.3 ± 9.0 mL/kg) followed by water (16.1 ± 2.5 mL/kg) and finally fluid deprived (24.5

Table 1. Burn injury alters WBC count, pH, glucose, and BEecf.

Time Point	WBC (1x10 ³ /μL)			pH			Glucose (mg/dL)			BEecf (mmol/L)		
	Fluid deprived	Water	ORS	Fluid deprived	Water	ORS	Fluid deprived	Water	ORS	Fluid deprived	Water	ORS
BL	20.52±1.43	19.03±0.67	16.60±1.18	7.42±0.02	7.44±0.01	7.42±0.01	66.95±7.84	72.36±9.68	51.01±8.34	5.60±0.51	6.80±0.49	4.17±1.74
6 h	#28.94±2.29	#28.15±2.93	#24.71±2.14	#7.51±0.02	7.47±0.01	#7.49±0.01	#545.83±282.49	517.61±280.27	293.13±77.35	#10.20±1.83	9.20±1.20	#8.33±1.20
12 h	#27.49±1.86	#26.56±2.61	#26.41±1.23	#7.50±0.03	7.47±0.02	7.46±0.01	211.15±33.01	305.20±80.17	#360.60±68.99	8.20±1.28	9.40±0.98	6.60±0.98
24 h	#25.51±1.88	#24.52±2.16	#25.48±1.67	7.41±0.01	7.44±0.01	7.41±0.03	180.03±28.60	161.23±20.52	159.98±22.60	#11.00±0.89	9.80±1.60	#8.25±1.38
32 h	#33.32±4.21	#32.90±2.92	#31.23±1.61	7.45±0.06	7.43±0.02	7.40±0.03	199.06±41.77	305.55±42.13	172.30±25.17	7.80±2.08	8.40±0.87	4.17±2.07
48 h	#25.74±3.34	#24.25±2.01	#24.71±2.16	7.41±0.03	7.44±0.01	7.41±0.01	257.10±95.20	98.90±4.78	115.81±25.34	9.25±1.60	8.60±1.16	6.67±0.88

White blood cell count, pH, glucose, and BEecf in blood samples were collected at baseline (BL) 6, 12, 24, 32, and 48 h post burn injury. Values are presented as mean ± SEM and a # indicates a significant ($P < 0.05$) difference from the BL value.

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±1.7 mL/kg; Fig 1B). Average GFR of the 48 h period was greatest in animals receiving ORS (71.5±13.6 mL/min) when compared to fluid-deprived (45.3±3.4 mL/min) and the water group (41.4±7.9 mL/min).

Plasma creatinine peaked at 6 h in all groups at an average of 1.67±0.07 mg/dL, indicating a moderate level of acute kidney injury by Kidney disease: Improving Global Outcomes stage 2 criteria (Fig 1C). However, the effect of enteral fluids again became apparent, wherein the fluid-deprived group (1.56±0.06 mg/dL) had greater ($P < 0.05$) circulating creatinine when compared with water (1.23±0.06 mg/dL) and ORS (1.30±0.09 mg/dL) groups at 48 h. Urinary creatinine mirrored that of plasma (Fig 1D). A steady increase in urinary creatinine following burn was noted in all animals, but at 48 h the levels began to diverge according to fluid intake. Specifically, both water and ORS groups reached maximum levels (235.3±40.7 and 198.0±18.5 mg/dL; respectively) by 24 h, while fluid deprivation resulted in a further increase of 266.9±45.3 mg/dL. At 48 h, significant differences ($P < 0.01$) in urinary creatinine levels were detected between animals receiving fluids (208.7±44.4 mg/dL 154.8±18.5 mg/dL for water and ORS, respectively) and those that did not (343.8±23.1 mg/dL).

These same temporal shifts are also seen with BUN. Maximal levels of BUN in the plasma were achieved 12 h following burn and also significantly diverged by 48 h (Fig 1E). At that time, plasma BUN in the water and ORS groups returned back to baseline levels (9.65±1.14 and 9.10±0.37 mg/dL respectively) whereas fluid deprivation led to significantly higher levels of circulating BUN (17.01±1.24 mg/dL; $P < 0.05$). Levels of urinary urea nitrogen were significantly greater than baseline by 24 h in all animals; however at 48 h the ORS (822.6±100.2 mg/dL) group was similar to the water (1,067.8±177.2 mg/dL) but significantly lower than the fluid deprived group (1,575.0±109.4 mg/dL; $P < 0.05$; Fig 1F).

Total protein in the urine was elevated by 32 h following burn in fluid deprived and water animals. At 48 h animals receiving ORS had lower levels ($P < 0.01$) of urinary protein when compared with fluid deprived animals (137.1±25.0 mg/dL vs. 289.3±43.4 mg/dL; Fig 1G). The group of animals receiving water (215.7±61.9 mg/dL) was intermediate to the fluid deprived and the ORS groups and therefore not significantly different between either treatment group at 48 h.

Enteral resuscitation prevents vasoconstriction of the renal artery

To assess renal perfusion and artery diameter, CT angiograms were taken at baseline and 48 h. Representative CT images from animals in each treatment group visually depict changes in the renal perfusion and vascularization (Fig 2A). Fluid deprivation led to a significant reduction in renal artery diameter, whereas enteral resuscitation with water or ORS

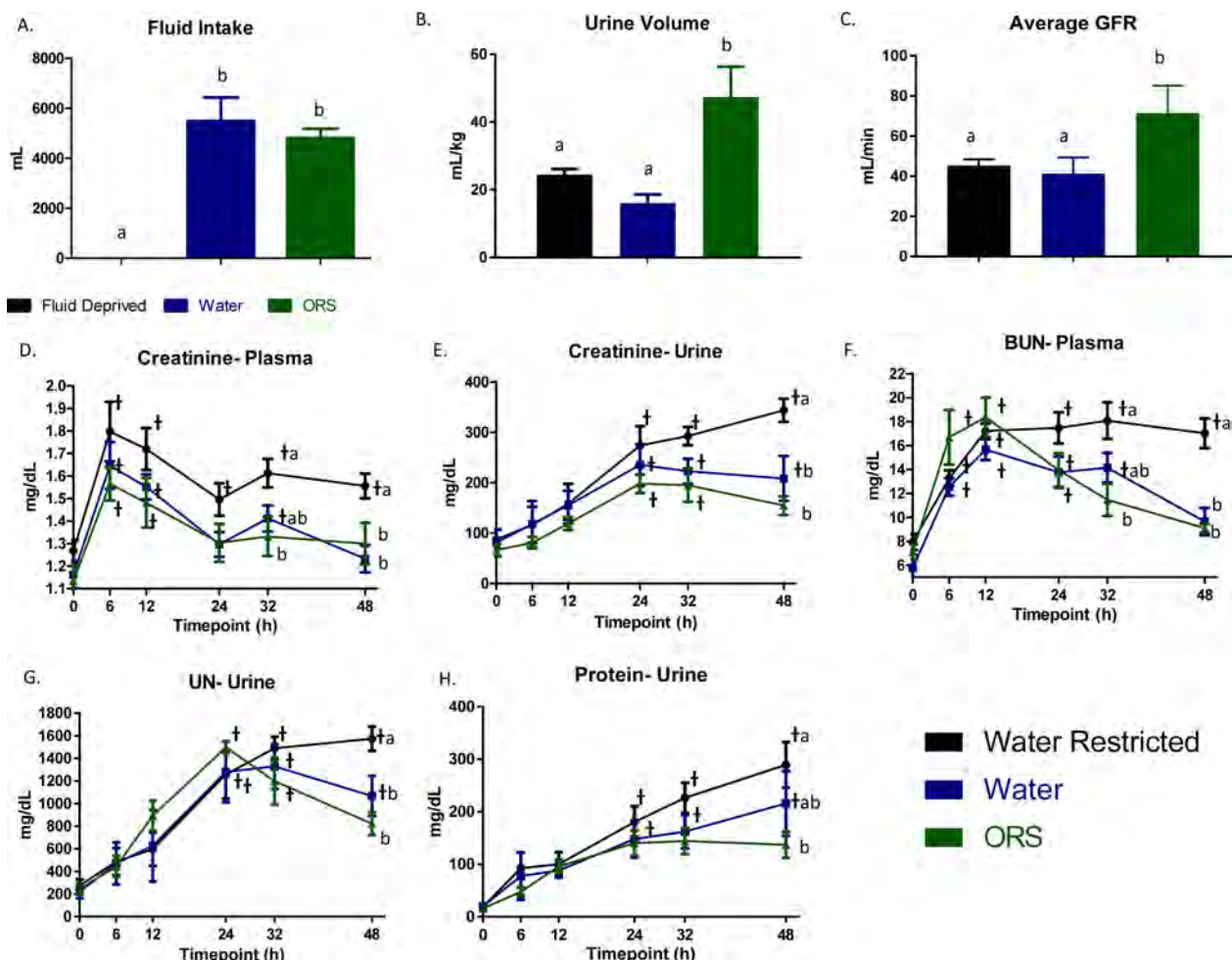


Fig 1. ORS increases urine output and positively alters burn-induced biochemical markers. Total fluid intake (A) urine output volume (B), and (C) Average glomerular filtration rate (GFR) throughout the duration of the study. Levels of creatinine (D, E) and urea nitrogen in the plasma (F) and the urine (G). Urinary protein (H). Means $\pm$ SEM with a different superscript are significantly different ($P < 0.05$) between treatments for indicated time point and a † indicates a significant ($P < 0.05$) difference from the BL value.

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maintained it (-1.4 ± 0.17 mm versus -0.44 ± 0.14 mm or -0.63 ± 0.20 mm respectively; Fig 2B; $P < 0.02$). Kidney volume significantly increased from baseline to 48 h post-burn in animals receiving water (20.0 ± 3.0 cm³), but did not change with fluid deprivation or animals consuming ORS groups (-0.38 ± 5.2 and 1.2 ± 5.5 cm³, respectively; $P < 0.02$; Fig 2C). Total kidney weight was similar in animals receiving water or ORS and statistically greater when compared with fluid deprivation (114.90 ± 3.48 g or 114.20 ± 4.50 g versus 93.58 ± 4.26 g respectively; $P < 0.001$; Fig 2D). Lastly, the wet-to-dry ratio was lowest in the fluid-deprived group (5.01 ± 0.27 g), and statistically higher in the water and ORS group (6.06 ± 0.27 g and 6.06 ± 0.38 g, respectively $P < 0.002$; Fig 2E).

Fluid deprivation increases kidney glycogen

Scores for tubular degeneration were 6.2 ± 0.8 , 7.2 ± 0.7 , and 7.3 ± 0.3 in fluid-deprived, water, and ORS groups, respectively, and were not statistically different from each other (data not shown). Other histological findings indicate glomerulonephritis hallmarked by microthrombi formation, synechia and parietal cell hypertrophy (Fig 3). Moderate to severe

A.

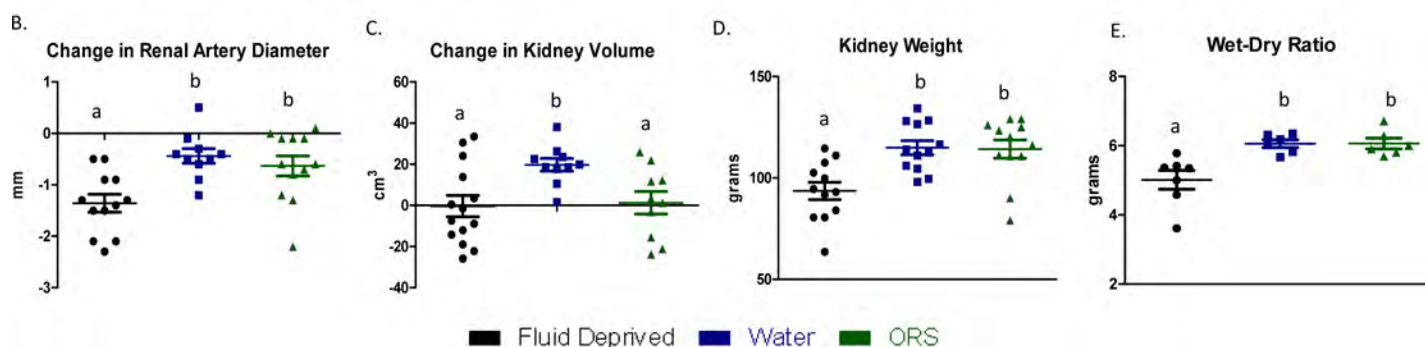
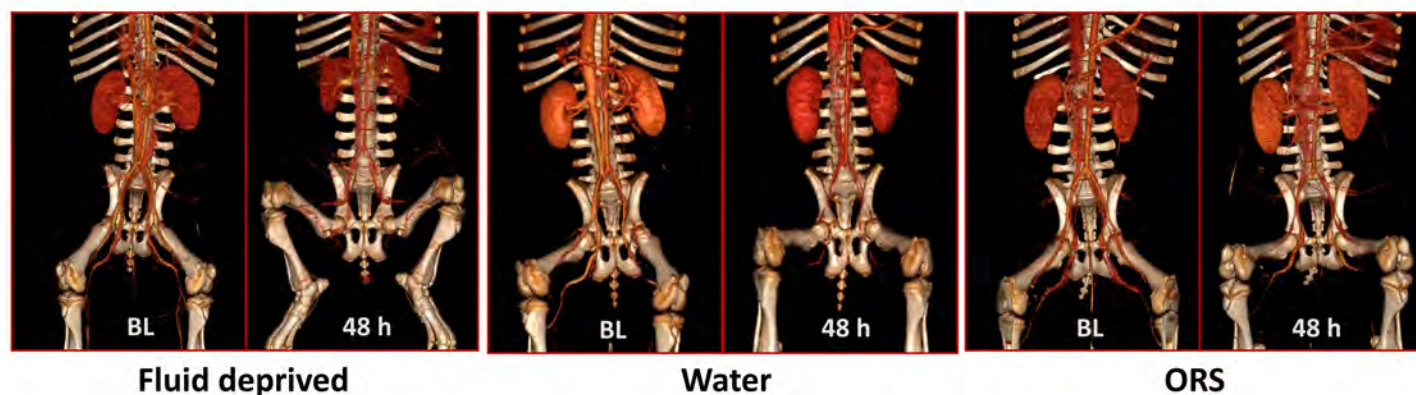


Fig 2. Enteral resuscitation prevents reduction in renal artery diameter. Computed tomography (CT) scanning (A) was performed pre-injury and immediately prior to euthanasia (termination of experiment 48 h). Renal artery diameter (B), kidney volume (C), weight (D), and wet:dry ratios (E) were quantified. For all parameters measured, changes from baseline to 48 h post burn are represented as mean $\pm$ SEM. Groups with different superscripts are significantly different ($P < 0.05$).

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glomerulonephritis was present in all animals in the water-deprived group, 2/6 animals in the water group, and 3/6 animals in the ORS group. Representative PAS staining reveals a darker intensity of staining in the fluid-deprived animals (Fig 3). After color deconvolution, the pink glycogen channel was normalized to the amount of nuclei (Fig 3B). As shown in Fig 3C, there is a significant increase in the glycogen content of kidneys from the fluid-deprived group when compared to the water and ORS groups ($P = 0.012$). While this may indicate a greater dependency of the kidney on gluconeogenesis as opposed to glycogenolysis, there was also greater glucosuria at 48h in the water deprived group (102.0 ± 39.8 mg/dL) than the water (39.6 ± 9.1) and ORS (22.8 ± 3.8 mg/dL) groups.

Inflammatory mediators are elevated in fluid-deprived animals

Pro-inflammatory and anti-inflammatory cytokine levels in plasma were quantified across time (Fig 4) and compared to averaged baseline levels. Expression of IL-1 β ($P \leq 0.019$), IL-6 ($P \leq 0.027$), and IFN γ ($P \leq 0.12$) was greatest in fluid-deprived animals regardless of time point; however, no time effect or differences between water and ORS were detected. Expression of IL-1 α significantly increased post-burn compared to baseline levels, and was also significantly highest in the ORS group at 6 h ($P < 0.002$). Granulocyte-macrophage colony-stimulating factor (GM-CSF) was lower than baseline levels in all animals, but was not significantly different among the groups. Circulating levels of the antioxidant enzyme SOD peaked 6 h following burn and then was significantly lower than BL by 48 h in all animals

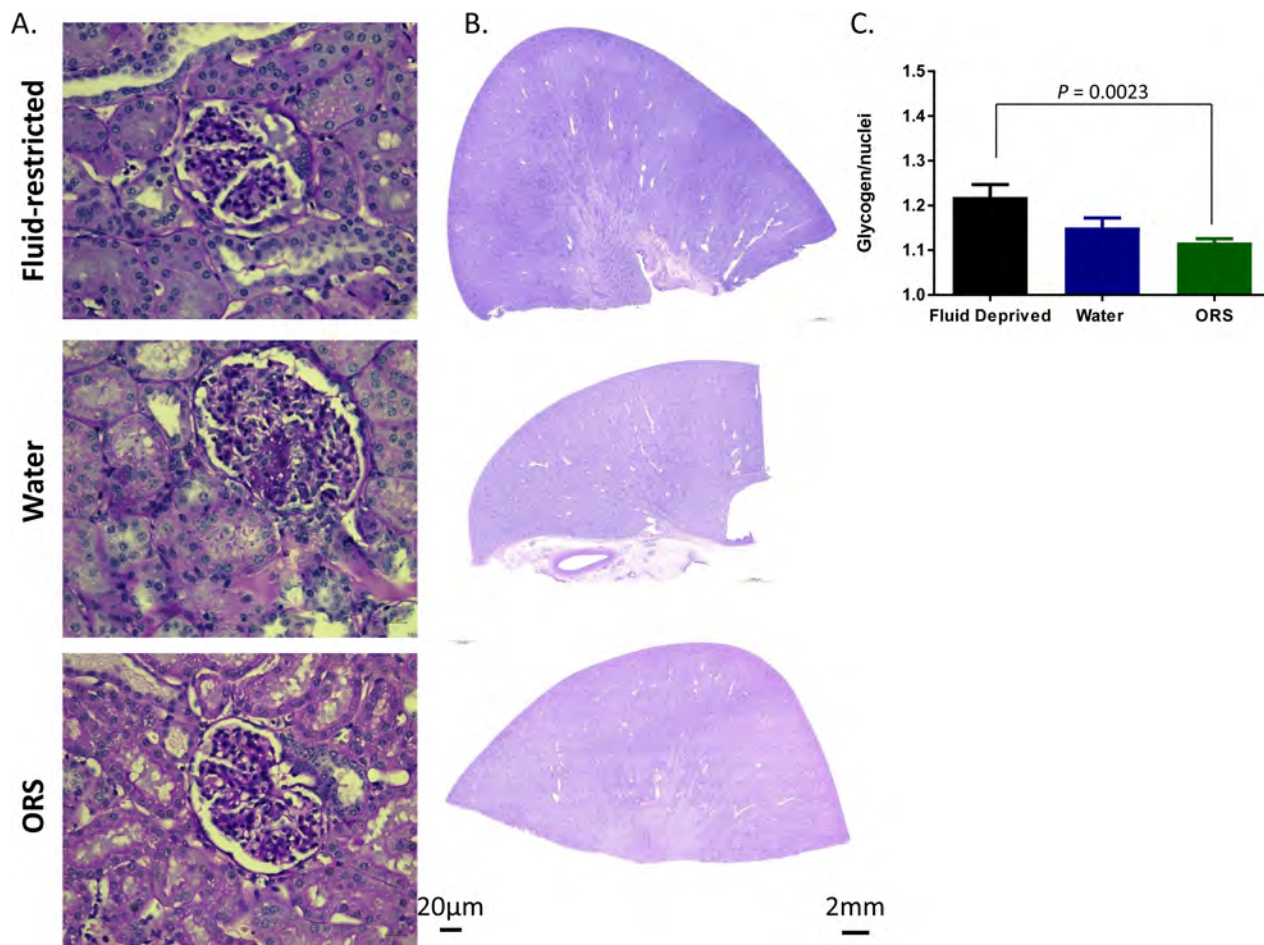


Fig 3. Renal glycogen content is greatest with fluid deprivation. (A) Representative H&E staining reveals hallmarks of glomerulonephritis in all groups, to include inflammatory cells (#), clotting with cell debris (*), parietal cell hypertrophy (arrow), and synechia (arrowhead) in all groups. Representation (B) and quantification of the color density reveals significantly higher glycogen content in the fluid deprived group compared with the water and ORS groups (* $P < 0.05$).

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($P < 0.05$; Fig 4F). Free hemin in the urine was elevated by an order of magnitude at 48 h in all animals (Fig 4G). However, urinary hemin was not significantly different among treatments, even when normalized to creatinine (data not shown).

Discussion

Severe burn injury elicits massive repartitioning of intracellular and extracellular fluids leading to circulatory dysfunction (i.e., vascular leak and edema) and subsequent organ damage. These hemodynamic fluctuations are treated with large volumes of IV fluids in an attempt to maintain adequate tissue perfusion, but this therapy may be associated with various co-morbidities and exacerbate tissue edema [15, 22, 23]. Animal studies [11] and clinical investigations [10, 12, 24, 25] dating as far back as the 1940s, demonstrate the effectiveness of enteral resuscitation in the treatment for burn shock. Indeed, several reviews on oral resuscitation in mass casualty care have suggested that provision of an enteral salt solution is effective for the treatment of burns [13, 26]. Despite this, there is a lack of evidence on its efficacy in ameliorating organ dysfunction after burn injury. The primary findings of this study are that AKI occurs in the acute time frame post-burn and enteral fluids (in the absence of IV resuscitation) have protective

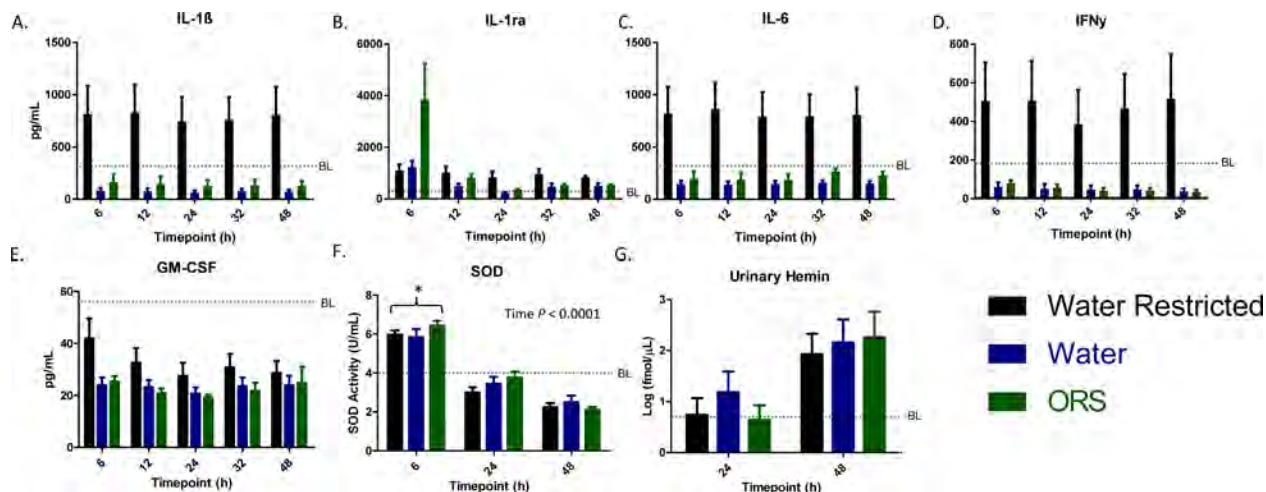


Fig 4. Fluid deprivation elevates circulating cytokines. Select circulating cytokines were quantified across all time points (A-E). Average of baseline values of all treatments is set as a reference line and bars represent mean ± SEM. Superoxide dismutase (SOD) activity in the plasma (F) and urinary hemin (G) was quantified at BL, 6, 24, and 48 h and presented as mean ± SEM. Asterisk denotes a significant difference ($P < 0.05$).

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effects on burn-induced renal damage/dysfunction, with the addition of salts conferring extra benefit.

Results presented herein compare two oral fluids and a fluid-deprived group after a 40% TBSA surface contact burn in swine. Following burn injury all animals presented with elevated clinical parameters for inflammation and AKI by 6 h. The efficacy of enteral resuscitation with ORS was most convincingly demonstrated with the improvement in GFR. Kidney recovery was also monitored via CT and showed oral fluids maintained volume and renal artery diameter. In the case of perfusion (i.e., Hounsfield units), no significant differences were detected among fluid deprived and enterally resuscitated swine (data not shown), perhaps due to reduced kidney weight. In this regard, these (Hounsfield) units may be a poor indicator of renal perfusion. Greater post-mortem kidney weights and wet-dry ratios in animals receiving fluids further demonstrated that enteral resuscitation maintained kidney perfusion. This could also be interpreted as development edema; however, significant edema was not seen with histology. Additionally, the former is further supported by a significant inverse relationship between plasma creatinine and kidney weight ($P = 0.04$; $r^2 = 0.17$) (data not shown), thus strengthening the relationship between perfusion and function.

While the ORS used in this study was selected based on its track record of use, there are several different rehydration solutions commercially available. Solution formulations vary, yet the basic composition consists of an isotonic or hypertonic sodium, chloride, and carbohydrate solution. The therapeutic efficacy of oral resuscitation with sodium lactate was demonstrated by Fox [10] who provided a preliminary detailed report on nine patients who improved with treatment. A more recent study showed a oral rehydration therapy containing rice-based (Cera-lyte®) carbohydrates reduced IV fluid requirements in burn patients [27]. While a clinical reduction in IV fluid requirements may circumvent the associated edema and co-morbidities, enteral fluids alone may be insufficient to ensure adequate end-organ perfusion. Still, as IV fluid requirements are driven off of urine output, the current report also indicates a reduction in IV fluids may be possible by administering ORS. Of note, target urine output is 0.5 ± 1.0 mL/kg/h and our groups span this range with ORS achieving urine output of 1.07 ± 0.21 mL/kg/h and fluid restricted at 0.52 ± 0.03 mL/kg/h.

The efficacy of ORS stems from the sodium-dependent absorption of glucose monomers in villous cells of the small intestine via the sodium-dependent glucose co-transporter (SGLT1) [28, 29]. Specifically, transport across SGLT1 requires Na^+/K^+ ATPase pump to create a downward sodium gradient for the movement of sodium ions and glucose across the apical membrane. While plasma glucose was temporally affected after burn injury (Table 1), this was not affected by the glucose present in ORS and could be indicative of feeding schedule. However, glucose present in the urine post-burn may corroborate this finding as a stress response to the injury. The ensuing osmotic gradient creates a hypertonic environment in the paracellular space [30], essentially replacing plasma volume [15]. The importance of solutes in these solutions is demonstrated in the current study by the failure of water to increase urine output and GFR. Still, further work is needed to optimize oral rehydration solutions. Solutions containing glucose polymers (e.g., rice-based carbohydrates mentioned above), short-chain fatty acids may potentiate absorption through the colon and the small intestine [28]. The effects of oral resuscitation fluid with pyruvate on the intestines have recently been investigated in 35% TBSA scalded rats. Intestinal absorption of sodium and water was increased with a pyruvate-supplemented ORS, although water alone was not tested [31]. Further, if large volumes of oral resuscitation conserve the gut mucosal lining, it would be noteworthy as disruption grants access of bacteria into circulation [13, 32]. For this study, animals did not develop bacteremia (even in the water deprived group). Whether this is because of maintained enterocyte integrity or the ability of the porcine immune system to sufficiently neutralize bacteria remains to be elucidated.

One of the primary tools clinicians use to drive the volume of IV fluids given is urine output, due to its estimation of end-organ (kidney) function. In this study ORS's superiority to water in increasing urine output and GFR would likely result in a decrease in the amount of IV fluids administered and potentially, edema. As indicated in Fig 1 the volume of fluid ingested, although slightly lower, was not different in animals given ORS, although they had nearly triple the urine output within the span of the study. Kramer et al. [13] reported a Chinese publication in a 30% TBSA burned canine model given orally a volume of a glucose, NaCl, and NaHCO_3 mix at an osmolality that is similar to ORS (347 vs. 331, respectively) or a hypotonic version according the Parkland formula (4 mL/kg/%TBSA). Similar to our data, improvements in urine volume excreted were noted with animals receiving the hypertonic solution. Moreover, a recent study randomized patients up to 20% TBSA to receive either enteral or IV fluids found that the only significant difference was higher urine output in the patients receiving enteral fluids. While the optimal type of enteral fluid for use in burns is unknown, the current study advocated for their use in improving creatinine clearance.

Similarly, the efficacy limit of enteral fluids in terms of volume also remains to be answered. The calculated volumes used in the current study (1.75 mL/kg/%TBSA) are slightly lower but comparable to IV guidelines given by the modified Brooke formula (2 mL/kg/%TBSA), and much less than the Parkland formula (4 mL/kg/%TBSA). The operational difference when examining the availability in austere environments lies in the fact that several liters of sterile IV fluids are heavy, while ORS is available in lightweight sachets that can be reconstituted with any potable water. Moreover, in disaster scenarios no special training is required to administer oral fluids. While these resource-limited environments were our initial motivation for examining enteral (but not IV) fluids, this potential treatment strategy has received strong interest due to the recent nation-wide shortage of IV fluids after the hurricanes in Puerto Rico. The questions of how much enteral fluid is effective, how long it might be effective, and in what injury severity it will be effective, remain unanswered. The volume of ORS used in this study is far below the absorptive capacities of the intestine, which is reported to be around 20 L/day [33]. This amount is approximately equal to Parkland estimations for a 70-kg individual sustaining a 70% TBSA

injury. Certainly in this case, IV resuscitation would be necessary, although total IV requirements could potentially be reduced with administration of enteral fluids.

In this regard, while large volumes of IV crystalloid is the standard of care, it has been shown that too much crystalloid is detrimental in burn patients (i.e., fluid creep) [34]. For example, large volumes of crystalloids can result in acute respiratory distress syndrome, compartment syndromes and MOD [35–37]. Crystalloid use was promulgated in the surgical literature for decades with studies done supporting mortality benefits for 'supranormal' hemodynamic parameters [38–40]. However, crystalloid is becoming increasingly recognized as a detrimental fluid because it is acidic, pro-inflammatory, and results in hemodilution. As such, in other forms of trauma (e.g., hemorrhagic shock) there has been a recent movement away from massive crystalloid resuscitation to other alternatives, or more moderate volumes of crystalloids. It would be interesting to see if IV fluid administration produced pro- or anti-inflammatory effects in this model, as enteral fluids (both water and ORS) reduced cytokine levels fairly non-specifically. However, ORS was able to increase the anti-inflammatory IL1ra and the antioxidant SOD when compared to the other groups at 6 hours. While this indicates that enteral fluids may buy extra time during triage or transport of patients, the effects of IV fluids on inflammation in this model remains the province of future investigation.

While this transition away from crystalloids in hemorrhagic shock patients took decades, it was logical given that blood was being lost, and should be replaced by whole blood [41–43]. While burn patients also have massive fluid losses, there is no direct blood loss per se, which is the justification for crystalloid infusion. Aside from transepidermal evaporation, there is massive fluid and protein shifts out of the intravascular space into visceral organs, which may explain a trend toward resuscitation with plasma [44]. To combat this, the contents of enteral fluid may be formulated to drastically alter the absorptive capacity of the small intestine. For example, one group has utilized ORS containing different compositions of amino acids, and shown that this affects ileal absorption of carbohydrates, amino acids, and electrolytes in radiation injury [45, 46]. Taken together, much more research needs to be done in order to realize a closer approximation to the fluid lost in the thermally injured population.

Limitations of this study include the acute nature of these experiments, with no indication on the long term effects of ORS. For example, we observed no critical imbalances in plasma electrolytes in animals receiving ORS, but cannot predict the effects of long-term administration of ORS (S3 Fig). Kidney tissue was harvested and processed at only one timepoint (48h) to evaluate the acute resuscitation period. Another limitation is that IV fluid administration which is the current standard of care (albeit with substantial variation in resuscitation protocols) was not included as a treatment group. While it is hard to imagine inferiority of IV fluids to enteral fluids, it also may be difficult to prove the opposite in this model, as enteral fluids alone sufficiently supported urine output and returned creatinine values to baseline. Regardless, future investigations into the efficacy of different types and volumes of IV fluids are needed. Although the severity of injury and demographics/ compliance of our subjects were uniform, future research could also explore volumes and types of both IV and enteral routes of administration to identify which patients may benefit from either route. Finally, it is highly unlikely for a burn victim to be deprived IV fluids and other definitive clinical care except potentially under austere military or wilderness environments or civilian mass casualty situations, where supplies may be extremely limited.

Conclusions

Data presented here suggest that enteral resuscitation is efficacious with regard to burn-induced renal dysfunction, and that inclusion of salts (i.e., ORS) provides additional benefit.

These results will help inform the design of clinical studies in humans to assess its safety and efficacy. Not only could this save lives in prolonged field care, mass casualty, or wilderness medicine scenarios, but its incorporation in definitive clinical care may reduce overall IV fluid requirements and the ensuing complications of resuscitation of the extravascular space. In patients receiving nasogastric tubes, this strategy could easily be incorporated into routine care. Future research should explore the enteral route for resuscitation and compare outcomes to the current standard of care.

Supporting information

S1 Fig. ARRIVE Guidelines.

(PDF)

S2 Fig. Veterinary support branch health and behavior check form. Veterinary technicians are required to monitor animal health and behavior daily of all animals (i.e. healthy and injured) on site. Technicians are trained to spend time with each individual animal to inspect and monitor well-being. This health and behavior check is independent of animal monitoring performed by research group. If abnormal animal behavior is recorded by trained staff, the head technician and/or veterinarian is notified for additional care.

(PDF)

S3 Fig. Swine pain and distress assessment score sheet. Parameters according to swine behavior and activity are monitored and reported following injury. Research team completes assessment and records results, if unsatisfactory behavior or severe complications are seen on call veterinarians are consulted for additional care.

(PDF)

S4 Fig. Plasma electrolyte levels following burn injury. (A) Sodium, (B) Potassium, and (C) Chloride levels in plasma following burn injury at 0, 6, 12, 24, 32, and 48 h in fluid deprived, water, and ORS treated swine. Means $\pm$ SEM with a different superscript letter are significantly different ($P < 0.05$) between treatments for indicated time point and a † indicates a significant ($P < 0.05$) difference from the BL value.

(TIF)

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Author Contributions

Conceptualization: Belinda I. Gómez, Kevin K. Chung, Leopoldo C. Cancio, David M. Burmeister.

Data curation: Belinda I. Gómez, Matthew K. McIntyre, David M. Burmeister.

Formal analysis: Belinda I. Gómez, Matthew K. McIntyre, Kevin K. Chung, David M. Burmeister.

Funding acquisition: Leopoldo C. Cancio, Michael A. Dubick, David M. Burmeister.

Investigation: Belinda I. Gómez, Matthew K. McIntyre, David M. Burmeister.

Methodology: Belinda I. Gómez, Matthew K. McIntyre, David M. Burmeister.

Supervision: Kevin K. Chung, Leopoldo C. Cancio, Michael A. Dubick, David M. Burmeister.

Validation: Belinda I. Gómez.

Writing ± original draft: Belinda I. Gómez.

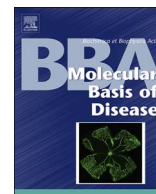
Writing ± review & editing: Belinda I. Gómez, Matthew K. McIntyre, Jennifer M. Gurney, Kevin K. Chung, Leopoldo C. Cancio, Michael A. Dubick, David M. Burmeister.

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Molecular mechanisms of trauma-induced acute kidney injury: Inflammatory and metabolic insights from animal models[☆]



David M. Burmeister, Belinda I. Gómez, Michael A. Dubick*

United States Army Institute of Surgical Research, 3650 Chambers Pass, JBSA Fort Sam Houston, TX 78234, USA

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ABSTRACT

Trauma-induced acute kidney injury (AKI), such as after hemorrhagic shock (HS) or burn, remains a significant problem in the intensive care unit and is associated with increased mortality. The pathophysiology that drives AKI post-trauma is multi-factorial, and includes both inflammatory and metabolic alterations. Identifying the systemic profile that contributes to AKI is crucial not only for early diagnosis, but also for identifying treatments that improve kidney function and maintaining long-term patient health. In an effort to elucidate this molecular pathophysiology researchers have utilized a variety of animal models including chemically-induced (i.e., cis-platin), blocking renal perfusion (i.e., arterial clamping) and inducing burn or HS. As the latter burn and HS models are unequivocally applicable to studying AKI in the context of traumatic injury, this review will summarize the inflammatory and metabolic insights associated with AKI gained with these animal models. Moreover, novel therapeutic strategies brought forth with these models will be discussed.

1. Introduction

Traumatic injuries such as hemorrhagic shock (HS) and burns can elicit a systemic cascade of metabolic and immunologic alterations that negatively affect distal organs [1]. The kidney is no exception, and HS or burn-induced acute kidney injury (AKI) is a common abrupt deterioration in kidney function that remains a significant problem in the intensive care unit, and complicates the prognosis of patients. This reduction in kidney function not only presents with immediate consequences during the initial hospitalization, but also leads to increased mortality and likelihood of progression to chronic kidney failure [2]. Over one quarter of patients with > 10% total body surface area (TBSA) burns develop AKI [3,4], and numerous studies in burn patients have shown that the presence of AKI post-burn is associated with significantly higher mortality compared to burn patients without AKI [3,5–8]. For example, a recent study showed patients with > 20%

TBSA burns have a mortality rate of 19.9% which triples (62.4%) in patients who develop AKI [2]. Similarly, in cohorts of critically ill patients with various traumatic injuries including HS, diagnosis with AKI was associated with significantly higher in hospital mortality [9].

The pathophysiology that drives HS and burn-induced AKI is multi-factorial and is not well understood. Thus, identifying the systemic alterations that contribute to AKI is crucial for early diagnosis, maintaining patient health and long-term kidney function. Many unanswered questions remain about the complex physiological response to trauma that propagates AKI in patients; therefore animal models are vital because they provide the ability to standardize the injury and collect multi-disciplinary data that address potential mechanisms involved. The purpose of this review is to provide a comprehensive overview of what animal models have taught us about the immunologic and metabolic physiological events that drive AKI in HS and burn trauma. This includes a discussion on the history of defining AKI in

Abbreviations: HS, hemorrhagic shock; AKI, acute kidney injury; TBSA, total body surface area; GFR, glomerular filtration rate; BUN, blood urea nitrogen; RIFLE, Risk, Injury, Failure, Loss of kidney function, End-stage kidney disease; ADQI, Acute Dialysis Quality Initiative; AKIN, Acute Kidney Injury Network; KDIGO, Kidney Disease Improving Global Outcomes; MOD, multiple organ dysfunction; IL, Interleukin; MCP1, monocyte chemoattractant protein 1; MAP, mean arterial pressure; HIF, hypoxic inducible factor; VEGF, vascular endothelial growth factor; ROS, reactive oxygen species; HO, heme oxygenase; KO, knockout; RNS, reactive nitrogen species; SOD, superoxide dismutase; NO, nitric oxide; NF- κ B, nuclear factor κ -B; TNF- α , tumor necrosis factor- α ; AQP, aquaporin; GSH, glutathione; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substances; MPO, myeloperoxidase; LPS, lipopolysaccharide; TLR, toll-like receptor; TRAIL, TNF-related apoptosis-inducing ligand; DAMP, damage-associated molecular patterns; PAMP, pathogen-associated molecular patterns; HMGB1, high-mobility group box 1; MAPK, mitogen-associated protein kinase; Akt, protein kinase B; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; NOX, NADPH oxidase; EPO, erythropoietin; IKK, I κ B kinase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; MPTP, mitochondrial permeability transition pore; MSC, mesenchymal stem cell; CsA, cyclosporine A

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* Corresponding author.

E-mail addresses: david.m.burmeister3.civ@mail.mil (D.M. Burmeister), belinda.i.gomez.ctr@mail.mil (B.I. Gómez), michael.a.dubick.civ@mail.mil (M.A. Dubick).

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trauma, as well as an analysis of the appropriateness of available AKI animal models. Finally, we include a discussion of promising therapies for the prevention and treatment of HS and burn-induced AKI.

2. AKI and trauma

While reduced glomerular filtration rates (GFR) present unequivocal evidence of renal dysfunction a number of surrogates exist, including novel potential biomarkers [10]. Traditionally, the clinical standards for diagnosis of AKI have been serum creatinine and blood urea nitrogen (BUN), however this has progressed to more sophisticated scoring systems that take into account urine output [11,12]. AKI has also been referred to as acute renal failure and has been difficult to quantify because of the multiple definitions of AKI found in the literature. In the last decade, clearer standardized scoring systems have been implemented to provide a foundation for consistency in all studies, whether investigational/animal, observational, retrospective, or prospective. One of these classification schema termed RIFLE (acronym for: risk of kidney dysfunction, injury to the kidney, failure of kidney function, loss of kidney function, and end-stage kidney disease) was the first attempt by the Acute Dialysis Quality Initiative (ADQI) to standardize AKI [13]. Additionally, the Acute Kidney Network (AKIN) modified the RIFLE system into three AKIN stages to define AKI more strategically [14]. Specifically, while both of these definitions utilize serum creatinine and/or urine output, the AKIN definition allows for the incorporation of small rises in serum creatinine if they occur within a 48 hour period.

More recently in 2012 these scoring systems of acute kidney dysfunction were combined by Kidney Disease Improving Global Outcomes (KDIGO) group [15]. The severity of AKI according to KDIGO classification is diagnosed by an AKI staging system that distinguishes the state of injury based upon the rise in serum creatinine and fall in urine output. Three criteria can be used to diagnose AKI stage I: 1) serum creatinine levels rise at least 0.3 mg/dL, 2) creatinine increases 1.5 to 1.9 times from the initial measurement or 3) urine output is < 0.5 mL/kg/h for 6–12 h. Stage II requires elevated serum creatinine between 2.0 and 2.9 times from baseline or urine output is < 0.5 mL/kg/h for 12 h or greater. Finally, stage III is characterized by an increase in serum creatinine ≥ 3.0 times from baseline, or ≥ 4.0 mg/dL, anuria for > 12 h, or a urine output < 0.3 mL/kg/h for > 24 h. While the criteria that dictate the presence of AKI in these different classification systems are very similar, their use may be tailored depending on the indication. For example, in critically ill patients, the KDIGO criteria was shown to be more sensitive in identifying AKI than the other two systems, and was more predictive of in-hospital mortality than RIFLE criteria, but not AKIN criteria [16].

In the thermally-injured patient, AKI may be classified as “early” or “late” based on whether the time to onset was before or after day ~5. Early-onset AKI usually relates to the initial reduction in plasma volume (i.e., hypovolemia) which decreases tissue perfusion [17,18]. The resultant ischemia/hypoxia has a synergistic effect with systemic inflammation and manifests in several physiological impairments (hyperglycemia, elevated body temperature, muscle wasting, etc.) because of a generalized hypermetabolic state [19]. Late-onset AKI is often driven by sepsis or associated with multiple organ dysfunction (MOD). Left untreated, systemic inflammation is often associated with a cytokine storm and oxidant burst, and is a major determinant in the development of MOD that often produces lethal results [20]. Similar to early AKI in thermally injured patients, HS patients experience a reduction in cardiac output that leads to poor organ perfusion, and hypoxia as a result of an immediate loss in blood volume. In this regard, HS results in even more extreme hypovolemia compared to burns, which is also coupled with metabolic and immunologic alterations to provide the foundation for the onset of AKI.

Severe traumatic injuries result in an influx of pro-inflammatory markers (e.g., cytokines, other signaling molecules) that have the

potential to supplement clinical data to predict AKI and/or patient outcomes [21–23]. In trauma patients who initially survive their injuries, increased plasma levels of the cytokines interleukin-1 (IL-1), IL-6, IL-8, and monocyte chemoattractant protein 1 (MCP1) within the first 24 h of admittance were associated with AKI occurrence [24]. If prolonged, these elevated levels of pro-inflammatory cytokines contribute to muscle cachexia and elevated whole body protein turnover in thermally-injured patients [25]. Particularly for burn trauma, the associated hypermetabolism further exacerbates kidney dysfunction, which can be lethal if essential protein reserves are severely depleted [26]. This review will summarize the intertwined nature of the relationship between oxidative stress markers and the innate immune response to both HS and burn injury.

3. Animal trauma models studying AKI

Although, the impact of AKI varies based on trauma severity and other co-morbidities, identifying and understanding the molecular abnormalities responsible for this condition is vital for early diagnosis and treatment. In an effort to understand the post-traumatic molecular mechanisms behind AKI, researchers have employed a variety of animal models of traumatic injury. Generating standardized animal models to mimic a clinically relevant situation of burn or hemorrhagic shock is difficult. In fact, some have argued that the more researchers control variables in their models, the less clinically translatable the data become [27,28]. For example, in trying to understand the pathophysiology associated with reduced kidney blood flow many studies incorporate clamping of the renal artery which occludes blood flow completely. These animal models have been reviewed elsewhere, but may not induce the systemic alteration seen in HS trauma patients [29]. Uncontrolled HS animal models may more closely resemble the clinical setting however, reproducibility of the model may be challenging [27,30].

Similarly, while animal models of mechanical and chemical (e.g., cisplatin) induced AKI may afford limited insights into burn-induced early onset and late-onset AKI, respectively, neither recapitulates the complex pathophysiology of burn injury. In addition, one of the leading causes of AKI in critically ill patients is the potential for sepsis, and animal models of sepsis-induced AKI (e.g., cecal ligation and puncture) may reproduce only certain aspects of burn-associated AKI [31]. Also, the extra endotoxin stimulus of LPS/sepsis models may confound the molecular basis for AKI after burn alone. Taken together, the models discussed above will be referenced sparingly, with a focus on animal models of AKI due to burn or HS, and the inflammatory/metabolic insights generated with them.

Articles for this review were selected using two independent searches, one for HS and the other for burn. Criteria for including articles into this review after both searches can be seen in Fig. 1. The first literature search in PubMed using “acute kidney injury” and “hemorrhage” with “Other Animals” checked turned up 2182 results as of October 18, 2016. As discussed above, several of the articles excluded from this review utilized a model of kidney damage by clamping the renal artery to focus primarily on the damaging effects of reperfusion. Traditionally, animal models of HS incorporate fixed pressure, fixed volume or uncontrolled hemorrhage, with or without a secondary traumatic insult. In this PubMed search, models of HS primarily included fixed pressure, where mean arterial pressure (MAP) was maintained between 30 and 45 mm Hg for a period between 30 and 180 min in anesthetized female or male mice and rats. Following HS, animals are often resuscitated with their shed blood, lactated Ringer's, or normal saline. The most widely employed model is the rodent, while others have incorporated swine, dogs, and to a lesser extent rabbits and sheep. Swine models were primarily used for evaluating hemorrhage control and resuscitation [32–34] and rodent models for understanding signal transduction mechanisms. Additionally, rodent models were often utilized to screen various treatments to reduce the incidence or severity of

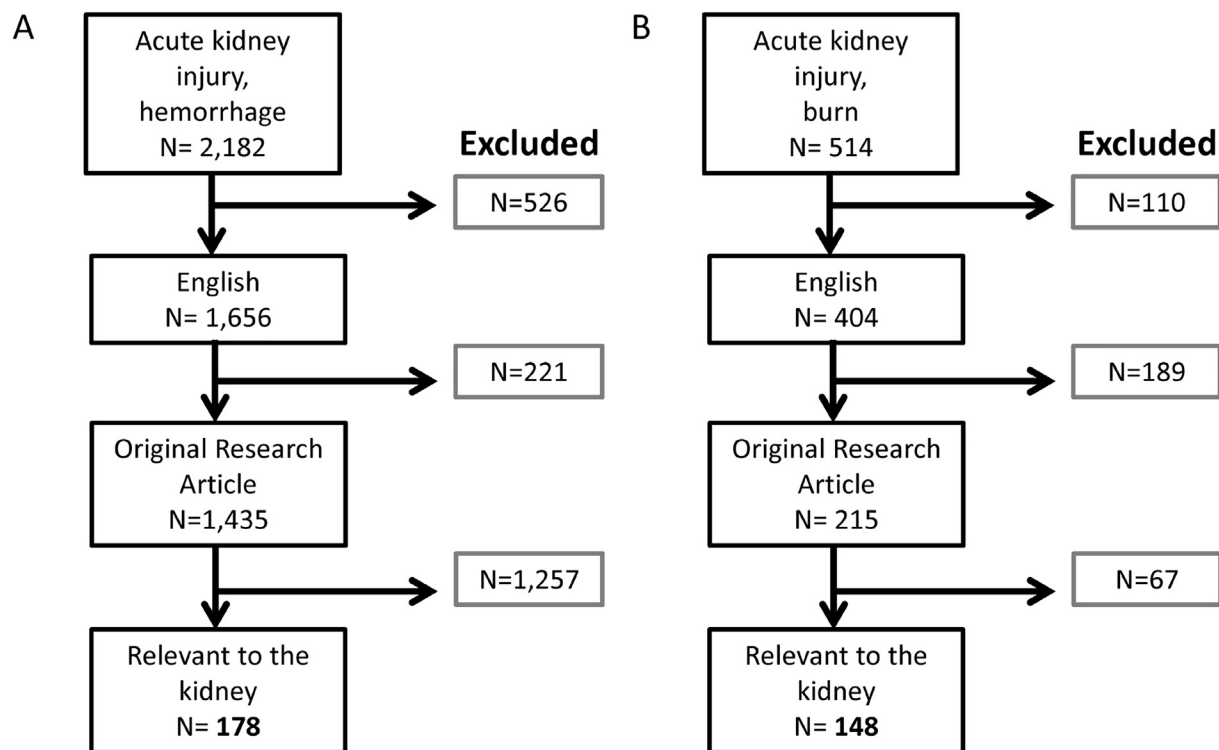


Fig. 1. Criteria for articles included in review from two individual PubMed literature searches using the key words: A) “acute kidney injury, hemorrhage” for retrieval of articles for hemorrhagic shock and B) “acute kidney injury, burn” for articles on burn injury.

AKI.

Animal models of burn-induced AKI have been utilized since the 1970s and initially were largely descriptive about renal morphology and cell proliferation [35–38]. To acquire articles for AKI in thermally-injured animals, “acute kidney injury” and “burn” were entered as search criteria into PubMed with 514 results as of October 26, 2016. Exclusion criteria for these studies are also shown in Fig. 1. The overwhelming majority of animal burn models reviewed herein incorporated a scald injury in the range of 20–40% TBSA on the dorsum of mice or rats. Following the immersion of the animal in boiling water for a few seconds, animals are often resuscitated intraperitoneally. In general, large animal burn models are regarded as superior clinical surrogates for burn injuries [39]. However, similar to HS, rodent models are usually utilized to examine molecular pathophysiology, likely due to the available tools (e.g., antibodies, knockout animals) available for the studies.

The type of evidence that investigators utilize to illustrate successful onset of AKI also varies widely. While KDIGO criteria of AKI are now routinely used in patients, they have not been widely applied in animal models of AKI. Using KDIGO criteria may be problematic because of varying normal levels of creatinine and urine output depending on the species [40]. For example, in rats baseline creatinine is normally around 0.3 mg/dL. Therefore, an increase of 0.3 mg/dL (which would be KDIGO stage 1 in humans) would actually constitute a doubling (i.e., KDIGO stage 2). Another limitation is that many of the animal studies are too short-term for the human scoring systems to be applied. Therefore, appropriately scaled values should be used for applying information used from animal models to the human condition to better link the animal studies with severity scores of human AKI. This strategy has recently been employed by us for defining systemic inflammatory response syndrome in swine to account for their increased body temperature and white blood cells counts under healthy conditions [41]. This also highlights an advantage of animal models in that baseline (pre-trauma) values can be obtained, which is almost never the case clinically.

Thus, when addressing the validity of animal models of AKI, the criteria used to define it are essential. For example, many studies describe the onset of AKI from a purely histopathological standpoint or a measurement of blood flow. The immunological and metabolic alterations in response to hypoxia contribute to the damage within the microvascular and tubular components of the kidney, which can be seen histologically. In one study following HS, several blood-filled and shrunken glomeruli were present and tubular necrosis was demonstrated by detached basal lamina [42]. Similarly, while models of electrical burns (and associated rhabdomyolysis) may be of benefit in studying AKI, only histological changes (glomerular congestion, proximal tubule degeneration) were shown without evidencing any functional changes [43]. This microstructural damage is not indicative of decreased renal function per se, and AKI can also occur in the absence of significant structural changes seen histopathologically [44]. Also, in the case of sepsis-induced AKI, renal dysfunction can occur concomitantly with normal, or even increased, blood flow, and global blood flow to the kidney may not be as important as localized areas of impaired perfusion [31]. As such, the overwhelming majority of the animal studies included in this review had creatinine and/or urine output data available.

The sections below summarize the information learned from animal studies on the mechanistic basis of HS and burn-induced AKI. Individual examples in burn models and HS models are given and are discussed together because of the significant overlap between inflammatory and metabolic responses in the two trauma subtypes. However, there are also instances of unique molecules in either scenario that will be noted. Many of these molecular players are summarized graphically in Fig. 2. This figure provides a unifying picture and will be referred to often, as it also depicts which signals are shared or unique to burn or HS injuries.

4. Oxidative stress in AKI

The signaling cascade that compromises kidney function in HS animals is initiated primarily by hypoperfusion resulting from the drop in

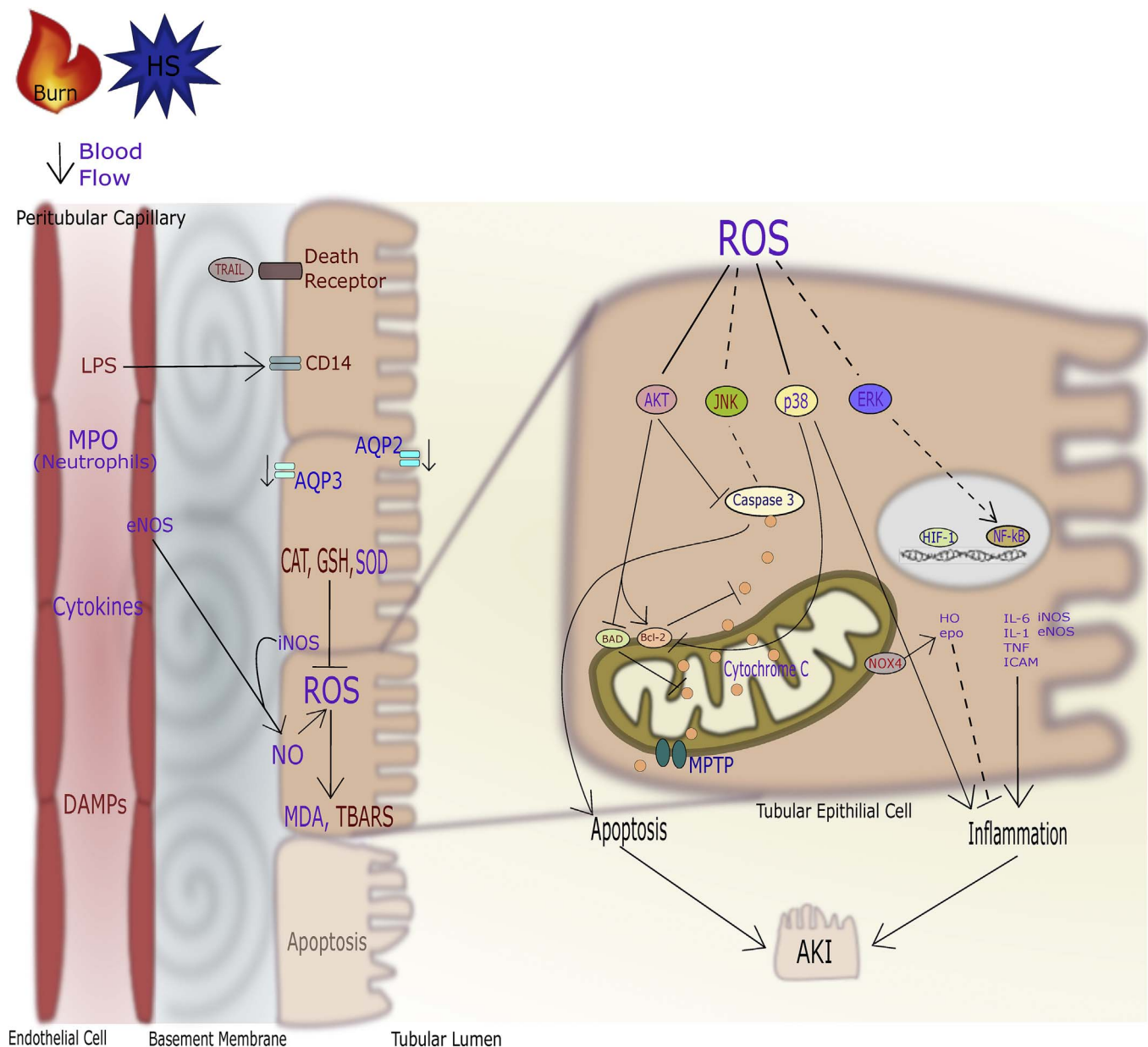


Fig. 2. Burn or hemorrhagic shock (HS) both result in reduced renal perfusion [17,18,60] that generates inflammatory and metabolic alterations in the tubule epithelia associated with acute kidney injury (AKI). Traumatic injury elicits an associated accumulation of reactive oxygen species (ROS) that result in activation of mitogen-activated protein kinases (MAPK) families such as c-Jun N-terminal kinase (JNK), p38, and, perhaps in burns, extracellular signal-regulated kinase (ERK) [75,120]. The antioxidant enzymes superoxide dismutase (SOD) [58,68], catalase (CAT) [73], and glutathione (GSH) [74] are also reduced resulting in further ROS accumulation and increased malondialdehyde (MDA) [58,74] and thiobarbituric acid (TBARS) levels. In burns, activation of the MAPK such as p38 enhances the expression of inflammatory cytokines [108,120] and induces apoptosis. Alternatively, activation of Akt positively regulates the promoter of cell survival, B-cell lymphoma 2 (Bcl-2), and negatively regulates the pro-apoptotic protein Bcl-2-associated death promoter (Bad). The result is the release of cytochrome C which is crucial because of the association with caspase and subsequent apoptosis [123]. In burn, cell death is also mediated by TNF-related apoptosis-inducing ligand (TRAIL) and death receptor 5 [90] and inflammation is, in part, driven by elevated CD14 in the absence of infection [85]. In HS, this may be mediated by opening of the mitochondrial permeability transition pore (MPTP) [60]. In both types of trauma, nuclear factor κ -B (NF κ B) is translocated to the nucleus and initiates the transcription of target genes that include interleukin 6 (IL-6) [58], IL-1 [24], tumor necrosis factor α (TNF- α) [58,141], and ICAM [58,75]. In HS, NF κ B also upregulates expression of nitric oxide synthases (NOS) that further exacerbates cellular oxidative stress [49,58]. In addition, hypoxic inducible factor (HIF) is stabilized and activated for the transcription of heme oxygenase (HO) [49,51], which catabolizes free heme [54]. Proximal tubule cells in HS are further compromised by disrupted water balance following reduced aquaporin 2 and 3 expression [64]. Finally, the role of damage-associated molecular pattern (DAMPs) in trauma is just being explored, and may reveal novel molecular targets [142]. Molecules in blue demonstrate regulation with HS, red denotes changes induced by burn, and purple denotes involvement in both HS and burn. Dotted lines indicate conflicting or minimal evidence is currently available.

blood pressure, and limited oxygen availability to the outer medulla. A minor fall in arterial blood pressure in a normally functioning kidney would elicit intrinsic signaling pathways (e.g., efferent arteriole constriction) to prevent adverse reductions in GFR. This autoregulation is an essential feedback mechanism to keep the glomerular filtration rate constant across a wide range of arterial blood pressures. However, the hypoperfusion that occurs after a major fall in MAP (i.e., < 50 mm Hg) stimulates several systemic and intrinsic mediators within the kidney.

Indeed, this may constitute the major difference between HS and burn injuries, as burn-induced AKI may occur in the absence of hypovolemia/hypoperfusion. Infusion of plasma from burned rats into healthy animals causes leukocyte activation and enhanced fluid extravasation and edema [45]. While the burn eschar itself may be a nidus for inflammatory substances, this study indicates that it is not necessary for the induction of systemic inflammation. Alternatively, others have postulated that the initial damage to the intestine may be the source of

cytokines that damage distant organs [46].

Generally, in response to low oxygen tension, cells target to stabilize and activate hypoxic inducible factors (HIFs), a heterodimer (HIF- α and HIF- β subunits) which regulate transcriptional activity to coordinate the adaptive response to hypoxia [47]. In renal artery clamp models HIF1- α protein was induced within an hour post-insult in whole kidneys of pigs, and collecting ducts, papillary tubular cells and interstitial cells of rats [47,48]. However, in HS mice mRNA expression of HIF- α , was not different from sham suggesting that HIF- α promptly responds to hypoxia by post-translational modifications [49,50]. These studies implicating hypoxia via increased levels of HIF within the kidney have not been reported in burn injury. While (especially from an inflammation standpoint) there are definitive examples of molecular overlap between HS and burn injuries (Fig. 2), the differences in hypoperfusion likely results in distinct targets in the two injury patterns.

Target genes of HIFs are the vascular endothelial growth factors (VEGFs), a family of signaling proteins that promote angiogenesis and vascular maintenance. In HS rats (MAP maintained at 35 ± 5 mm Hg) and pigs (MAP maintained at 40 mm Hg), kidney expression of these growth factors and their receptors is reduced however; lack of expression may be due to time of collection or severity of kidney injury [49,51]. Interestingly, in diabetic HS mice VEGF-2 receptor was reduced to a lesser extent than in non-diabetic HS mice [49]. These authors further suggested HS effects on the kidney were more severe in mice with type II diabetes as diabetic HS mice displayed elevated creatinine, medullary cellular fragmentation, and vacuolization, while they failed to induce AKI in non-diabetic HS mice (i.e., creatinine did not change). To date we did not find literature on expression of these receptors in the kidneys of thermally-injured animals yet, researchers have studied the effects of VEGF and other growth factors on burn wound healing [52].

Ischemia following hemorrhagic shock has resulted in free heme which contributes to the production of reactive oxygen species (ROS) and oxidative stress within the kidney [53]. As shown in Fig. 2, ROS have been reported to be an upstream mediator of many molecular responses in both burn and HS. To counteract the accumulation of ROS, another target of HIF is heme oxygenase (HO), an enzyme that protects cells from free heme through oxidative catabolism. In a rat HS model HO-1 mRNA expression in tubular epithelial cells was rapidly induced within 3 h following HS and reperfusion [54,55]. In HO-1 knockout (KO) mice caspase 3 was robustly increased, and kidney damage was apparent with renal artery clamping for 15 min [56]. Kidney damage was assessed by significantly elevated creatinine in HO-1 KO injured mice compared with shams. HO-1 expression (and serum creatinine) has also been shown to be increased within the kidneys of mice after 15% TBSA burn injury [57].

The generation of reactive oxygen and nitrogen species (ROS and RNS, respectively) has been associated with HS-induced renal dysfunction. Renal malondialdehyde (MDA) generation, reduction in superoxide dismutase (SOD) activity and induction of inducible nitric oxide synthase (iNOS) and subsequent nitric oxide (NO) production were shown after HS in rats [42]. ROS and NOS facilitate the translocation of the transcription factor nuclear factor κ -B (NF- κ B) from the cytoplasm to the nucleus, which elicits the inflammatory cascade. Once in the nucleus, NF- κ B initiates expression of iNOS [49,51], tumor necrosis factor α (TNF- α) [58–60] and interleukin 6 (IL-6); all elevated in rat kidneys within 2 h of HS and resuscitation [42]. iNOS and NO production are also generated during hypoxia in rat proximal tubules [58,61]. NO derived from iNOS propagates the inflammatory response and contributes to enhanced tissue damage. Additionally, endothelial nitric oxide synthase (eNOS) was capable of generating NO in the kidney 6 h following HS in pigs [51] but not in mice [49]. In contrast, hypoxia from hemorrhage in rats was associated with a 10-fold drop in NO and renal damage and dysfunction [62]. NO has also been shown to regulate water permeability via inhibition of vasopressin [63]. Additionally, HS further reduced the abundance of aquaporins (AQP), a

family of membrane protein water channels, in the kidney [64]. In principal cells of distal or collecting tubules, AQP2 in the luminal membrane and AQP3 in the basolateral membrane, but not AQP1 in the proximal tubule, was reduced following HS. However, whether NO acts via a direct mechanism in reducing water permeability through AQP has yet to be investigated. The status of renal aquaporins has been far less studied in burn animal models.

The kidney contains an abundance of polyunsaturated fatty acids, which makes it particularly vulnerable to oxidative stress regardless of trauma type. Numerous studies in experimental animals and humans have reported reduced antioxidant status related to thermal injury [65]. A rat model of extensive burn was utilized to provide initial evidence about the importance of free radicals in burn-induced AKI [66]. Since most free radicals have extremely short half-lives, luminol-mediated chemiluminescent reactions have been used as a proxy for ROS levels in the kidney [67]. In addition, it is commonplace to measure activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase, as well as reduced glutathione (GSH) in kidney tissues post-burn and HS. MDA and thiobarbituric acid-reactive substances (TBARS) as markers of ROS-induced lipid peroxidation have also become routine measurements in animal models. All of these are indicated in Fig. 2.

As an example, Gotoh et al. utilized 2 different levels of burn injury (i.e., 35 and 60% TBSA) to demonstrate that while mRNA levels of SOD increased in the kidney in a dose-dependent fashion, the amount of SOD protein was not elevated dose-dependently [68]. In addition, a series of investigations by Sener et al. was among the first to examine the effects of antioxidant therapy on ROS markers and kidney function (i.e., creatinine) post-burn injury [69–73]. These studies utilized a full-thickness scald injury in rats to examine the benefits of anti-inflammatory compounds such as melatonin, ginkgo biloba extract, and Rosiglitazone after 24 h. These results, along with others [74], established very clearly that the amount of MDA in the kidney increases post-burn, while GSH levels concurrently decreased. While the antioxidants tested consistently reversed these findings, the effects on kidney function were not as clear. Some antioxidants exerted a marked beneficial effect on creatinine levels [72] compared to others [71]. Moreover, despite consistent methods, the creatinine levels were not always increased to the same degree by the injury [73], highlighting the substantial challenge of variability in animal models of AKI that inherently comes with in vivo studies. A similar study performed by Wang et al. demonstrated that the increased MDA levels after HS in rats was reduced by treatment with the antioxidant crocetin [58]. These researchers concluded that although HS reduced total SOD, crocetin provided protection by elevating total SOD. Administration of hydrogen sulfide also neutralized reactive molecules and has been shown to reduce kidney injury scores in a HS + ischemia reperfusion (aortic cross clamp) pig model [51]; however, creatinine levels were not affected by hydrogen sulfide treatment at the time of animal euthanasia (6 h) [51]. Clinically, it seems that while targeting free radicals may show some benefit, any successful treatment for end organ damage needs to exert pleiotropic effects or will require some type of treatment cocktail.

5. Immunologic alterations in AKI

Related to the discussion on oxidative stress on the kidney in burn and non-burn trauma, many studies have also implicated altered levels of myeloperoxidase (MPO) in the kidney (Fig. 2) [71–73,75–77]. This enzyme is abundantly released by the neutrophil during the cells' respiratory burst and an enzyme assay is used as a marker of neutrophil activity within tissue. MPO generates hypochlorous acid which neutralizes bacteria, but also may cause oxidative damage in the host tissue. Infection is a serious consideration in burn patients; however animal models have proven that neutrophil levels rise even in the absence of infection [41]. Additionally, MPO positive cells in the kidney medulla, but not cortex, are elevated by 5 h following HS [78].

While the above studies suggest that neutrophil infiltration may be

an attractive target for AKI, most of the evidence to date has been associative or conflicting. In an ischemia/reperfusion model, knocking out an E-selectin ligand CD147 (affecting extravasation), was used to show that neutrophil expression of CD147 was necessary for the development of AKI [79]. As mentioned earlier, a major limitation of this study was the sole use of BUN as a marker of renal function. In contrast, depleting neutrophils in an ischemia/reperfusion model was only shown to reduce serum creatinine marginally [80], more in line with earlier studies showing no change at all [81]. To our knowledge, studies in burn animal models that target neutrophil infiltration have not shown efficacy, perhaps due to the time-dependent nature of neutrophils within the kidney [82]. Again, the etiology of AKI is likely of the utmost importance, as neutrophil depletion was protective of renal function in sepsis- [83] but not cisplatin-induced AKI [84]. Clearly, more research needs to be done to elucidate whether targeting neutrophil activation in HS- or burn-induced AKI is a viable option.

The innate immune system has also been implicated in AKI in other ways. Another rat model of 35% TBSA scald injury has shown that thermal injury itself in the absence of infection can result in markedly increased levels of endotoxin in several organs including the kidney [85]. In this study, lipopolysaccharide (LPS) binding protein RNA was elevated in the kidney at 12 h after the injury. Moreover, the CD14 receptor (which binds LPS) was also elevated in the kidney for 2 days, further implicating the innate immune system. However, this study failed to show a significant increase in serum creatinine, making the applicability of CD14 and LPS to burn-induced AKI unclear. Also intriguing, is the possibility of implicating toll like receptor-4 (TLR-4), as initial CD14 binding of LPS ultimately presents the ligand to TLR-4 [86]. It is well known that TLR-4 is upregulated in the kidneys of septic animals [87], but its role in hemorrhage and/or burn-trauma remains largely unstudied.

In general, studies of inflammation associated with traumatic injury have often centered on plasma or tissue levels of cytokines (Fig. 2). Elevated levels post-burn or HS have often been implicated with contributing to the development of MOD, including AKI. For example, in a 35% full thickness rat burn model the cytokine tumor necrosis factor TNF- α and TNF- α receptor 1 (TNFR1) mRNA and protein levels were determined in kidney and other organs. Specifically, despite no changes in serum creatinine or renal levels of TNF- α mRNA, a strong correlation was observed between serum creatinine and TNF- α R1 [46]. The authors suggested that the expression of TNF and its receptor could be involved in the development of MOD following burns. This was further supported by a report studying early enteral feeding after a scald burn in rats, which significantly improved creatinine clearance, and reduced blood concentrations of TNF [88], despite no changes in circulating endotoxin. In addition, another study revealed that their 35% burn injury produced some perturbations in renal function, as evidenced by significantly increased BUN [89]. However, treatment of burn injury with a monoclonal antibody neutralizing TNF had no effect on BUN but did reduce markers of liver or muscle injury. Leng et al. have recently reported a critical role for the interaction between TNF-related apoptosis-inducing ligand (TRAIL) and death receptor 5 (DR5) in the pathogenesis of AKI. In their mouse burn model, serum creatinine and BUN progressively rose over 24 h, and administration of soluble DR5 reduced creatinine, as well as alleviated apoptosis and injury score in the kidney [90]. Taken together these studies implicate the TNF family of proteins in AKI after burn.

Other inflammatory players such as, damage-associated molecular pattern (DAMPs) and pathogen-associated molecular pattern (PAMPs) are of great interest for AKI as they are filtered by the kidney and can trigger inflammatory responses and bioenergetics failure, but their association with AKI has not been studied in burn or HS animal models in detail. DAMPs and PAMPs have been implicated often in sepsis-induced AKI [91], and therefore may have a role in late-onset AKI related to infection. A protein of specific interest, high-mobility group box 1 (HMGB1) has been shown to be unregulated in rat kidneys (as well as

lung and liver) on both the mRNA and protein levels post-burn [91]; however this study made no attempt to determine AKI by creatinine or urine output.

As a critical regulator of the immune response that responds to both endogenous and exogenous patterns, the complement system is also a logical pathway to analyze. The complement system is implicated in AKI of various etiologies, which has been reviewed elsewhere [92]. While, to date, the evidence implicating the complement system in sepsis is more extensive than burn or HS, some studies may indicate pathway specificity. Importantly, the complement system is divided into the classical (C1), alternative (C3) and Lectin pathways. Previous studies in burn have utilized a 30% TBSA swine burn model to show that inhibition of C1 (classical pathway) prevents edema and bacterial translocation within the gut [93]. The same group showed C1 inhibition reduced capillary leakage and tubular degeneration within the kidneys [94]. Alternatively, C3 has been shown to be increased in burn wounds [95]. Kidney function was not assessed in these studies. However, it has been shown that in trauma patients, the alternative pathway predominates in the acute setting [96] and that the alternative pathway is responsible for tubular necrosis [97]. The alternative pathway has also been implicated in AKI in ischemia reperfusion models [97] which has also been reviewed elsewhere [98]. Despite this, inhibition of the classical pathway has been shown to reduce creatinine in a pig model of controlled hemorrhage [99]. In addition, in a swine hemorrhage model, marked renal tubular injury was observed that was less evident in animals treated with Decay Accelerating Factor, a classical and alternative pathway inhibitor [100]. Taken together, the information obtained from renal ischemia should be used to address the minimal data available on the role of the complement system in HS- and burn-induced AKI.

Altogether, while the effects of targeting one cytokine/DAMP/PAMP may be insufficient, it is also possible that non-specifically targeting all cytokines will not be advantageous as shown in a recent study of cytokine hemoabsorption in a swine burn and smoke inhalation model [101]. In that study, IL-1 β , IL-6 and IL-10 were removed across the Cytosorb™ membrane, but led to no significant improvements on survival or inflammation. Moreover, the AKIN criteria was used to show stage 1 AKI, however hemoabsorption did not reduce creatinine levels. Also, lipid-mediated resolution of inflammation has been postulated in cases such as sepsis. For example, a recent study used a rat model of burn and sepsis-like conditions (LPS injection) to show the improvements in renal pathological changes due to resolvin D2 [102]. However, the authors did not show any significant increase in creatinine due to their injury. In short, the prospect of altering the levels of these inflammatory signals in the post-burn kidney warrants further investigation and identifying inflammatory markers for effective therapeutic targets to reduce AKI remains elusive.

6. Mitochondrial damage and apoptosis

Fig. 2 illustrates the importance and implication of renal mitochondria in AKI after traumatic injury. Mitochondria are the linchpin organelle essential for maintaining normal cell bioenergetics and are present in both microvascular endothelial cells and the highly energy-dependent kidney tubular cells. The above-referenced inflammatory responses elicit a disruption in mitochondrial function that includes a loss of ATP production and reduces the respiratory control rate of mitochondria in the kidney [60]. In a HS rat model based on respirometry (i.e., the ratio of mitochondrial oxygen consumption in the presence and absence of ADP), mitochondrial function was reported to be significantly reduced post HS [60]. However, additional studies have observed mitochondrial dysfunction associated with higher creatinine levels post HS (however no causation was determined) [103]. Recently, multiphoton microscopy was used to demonstrate damaged mitochondrial structure and reduced mitochondrial membrane potential after ischemia reperfusion [104]. While respirometry has been used to

explore dysfunction in other organs post-burn [105], the function and structure of renal mitochondria post-burn is less studied. Considering burn-induced hypermetabolism, the high energy needs of the kidney for solute transfer makes this organ particularly susceptible to mitochondrial damage.

Another aspect of HS and burn-induced AKI that has been of recent interest, relates to mitochondrial-induced apoptosis (Fig. 2) [106]. Certainly, the process of mitochondrial driven apoptosis is intimately tied in with oxidative stress and inflammation, and it is sometimes difficult to distinguish which processes are potentially causative. The molecular mechanisms behind mitochondrial-related apoptosis seen post-trauma is being explored only recently. For example, the role of mitogen-associated protein kinases (MAPK) pathway such as p38 in mitochondrial apoptosis has been identified [107]. Implication of this MAPK pathway in regulation of inflammatory mediators in burn-induced AKI has also been shown [108]. These authors showed that an inhibitor of p38 MAPK reduced the rise in BUN seen post burn in rats, and ameliorated histological evidence of renal damage. Similarly in rats, treatment with FR167653, an inhibitor of p38 MAPK activation reduced mRNA expression of TNF- α , IL-1 β , and prevented the expected increase in serum creatinine after HS [109]. More recently, several studies have examined this p38 pathway even further while simultaneously elucidating additional molecular mechanisms. For example, Guo et al. showed that serum creatinine levels tripled after 40% TBSA scald injury in rats, which was reduced with the anti-oxidant astaxanthin [110]. This anti-oxidant partially blocked lipid peroxidation (MDA) and restored levels of SOD and catalase, while preventing apoptosis. Cytochrome C, caspase 3, and caspase 9 were all significantly increased post-burn, and subsequently reduced with astaxanthin, suggesting reduction in mitochondrial damage. Molecularly, this was done by augmenting the protective phosphorylation of protein kinase B (Akt) and also increasing phosphorylation of bad (Bcl-2-associated death promoter homologue). As Fig. 2 illustrates, the bcl-2/bad axis of proteins is intimately involved with whether stressors results in cell survival or mitochondria-driven apoptosis [111], including in AKI [112].

It should be reiterated that many of the molecular players listed above are inseparable from the inflammatory molecules mentioned in the previous section. As Fig. 2 depicts, many of these signaling pathways are intertwined with one another. A prime example of this is the p38 MAPK pathway (along with other protein kinases), which not only respond to stress signals, but are also involved in cytokine production [113]. This remains true in several renal diseases, including AKI, wherein p38 inhibition prevents inflammation and apoptosis [114]. This has also been shown in a murine burn model, where topical application of p38 inhibitors reduces epithelial apoptosis and systemic inflammation [115,116]. New upstream targets are being identified that may act on multiple MAPKs for even more pronounced pleiotropic effects [117]. Certainly, more research into leveraging these pleiotropic effects for treating burn and/or HS-induced AKI is warranted.

The temporal aspects of these different molecular players should also be considered, as previous studies have shown that alterations of these key molecules in the kidney can last for several days [118,119]. In those studies, burn-induced AKI was associated with elevated renal concentrations of bcl-2 and major histocompatibility complex class I-chain antigen, which were sensitive to ulinastatin. While absolute levels of proteins like bcl-2 may be revealing, ultimately the phosphorylation status of these regulatory proteins represent their active form. In this regard, another study by Guo et al. confirmed burn-induced AKI in their rat model out to 72 h, and also further elucidated the mechanisms involving MAPKs [75]. Similarly, they found burn-induced alterations in serum creatinine, oxidative stress markers, apoptosis, and levels of cytokine expression. Importantly, time-dependent increases in the phosphorylation state of apoptosis-related MAPKs such as Akt, p38, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) was also shown. Moreover, there was an increase in the overall amount of the transcription factor NF κ B, further linking these

pathways to inflammation post-burn (Fig. 2). These changes were ameliorated (or augmented in the case of the anti-apoptotic signal Akt) with hydrogen-rich saline resuscitation [75]. Considering the ease of implementing this resuscitation fluid in practice, further research in this area should be considered.

Another study by Feng et al. investigated similar molecular mechanisms over the same 72 h time frame post-burn [120]. Increased creatinine, BUN, apoptosis (with caspase cleavage) and indices of oxidative stress (hydrogen peroxide and TBARS) were apparent over this time, as was a decrease in manganese-SOD (the mitochondrial-specific SOD isozyme). While this study found similar changes in P-Akt and P-p38, they did not find any differences in the phosphorylated JNK and ERK in contrast to the previous study. Despite these discrepancies, both studies implicated mitochondrial-driven apoptosis pathway in burn-induced AKI, and implied a potential therapeutic benefit from p38 MAPK inhibitors. Moreover, the Feng study [120] also found a 3-fold increase in NADPH oxidase 4 (NOX 4) expression in kidney tissue. This is an interesting finding, as NOX 4 has been postulated to be an “oxygen sensor” in renal mitochondria, and may lead to production of erythropoietin in the kidney [121]. Interestingly, it has been shown that pretreatment with recombinant human erythropoietin (rhEPO) significantly reduced HS and burn-related increases in serum creatinine levels [122,123].

The above mentioned study by Feng et al. [120] not only illustrated that renal failure at 72 h post-burn may relate to ROS-mediated late inhibition of Akt phosphorylation (Fig. 2), but that administration of Akt inhibitors exacerbated renal damage. The role of Akt pathway in AKI was also implicated in animal models utilizing renal clamping and reperfusion injury, but not as extensively as in models of HS. Within 30 min Akt and Bad phosphorylation was increased in kidneys from ischemic mice [124]. Conversely, Akt phosphorylation in the kidney was not increased in HS rats 4 h after reperfusion when compared to sham animals [78]. These differences may relate to observations that phosphorylation events occur rapidly and therefore signal may have been lost by 4 h following trauma, indicating that timing of therapeutic intervention may be relevant. These authors did indicate that HS induces cross talk between Akt and NF- κ B signaling pathways by inhibiting the I κ B kinase (IKK) complex with 1 mg/mL/kg i.v. infusion of the molecular inhibitor IKK16 (Fig. 2) [78]. Treatment with IKK16 significantly enhanced Akt phosphorylation and activity in the kidney of HS rats. The IKK complex phosphorylates I κ B α thereby facilitating the release and translocation of NF- κ B to the nucleus. They also demonstrated that IKK16 reduced creatinine and MPO positive cells in the kidney after HS, but this has not been identified after burn injuries.

Caspase expression as mentioned previously is induced with burn and HS and is often quantified to demonstrate activation of cell apoptosis [123,125,126]. In trauma and HS rats the amount of TUNEL positive cells in the kidney paralleled that of relative caspase 3 and 9 expression [127]. More recently, perturbations in mitochondrial function may also be attributed to open mitochondrial permeability transition pore (MPTP) that deregulate the permeability of the inner mitochondrial membrane as seen in HS rats [60]. Opening of MPTP induces cell death through the release of cytochrome c and subsequent caspase activation [128]. Taken together, these studies identify mitochondria, apoptosis and MAPK and Akt pathways related pathways as potential novel targets for treating trauma-induced AKI [117].

7. Therapeutic interventions

Fluid resuscitation is one of the basic treatment strategies for both traumatic hemorrhage and burn trauma [129,130]. Although the majority of studies in animal models of HS have focused on the effects of various fluid resuscitation formulations on hemodynamics and survival [131], a few have included renal function. In a study of asymptomatic pneumonia after HS, pigs resuscitated with LR had significantly lower urine output compared to healthy pigs, suggesting some degree of renal

failure [132]. In addition, the effect of hemorrhage and hypertonic fluid resuscitation in dehydrated animals was postulated to compromise renal function. However, no significant effects were observed on glomerular filtration, renal blood flow or filtration fraction compared in dehydrated animals resuscitated with 2 different fluids after HS [133]. A study of hypertonic fluid resuscitation in a 70–85% scald burn model also did not observe a difference in urine output compared to LR treated sheep [134]. Furthermore, despite an increase in renal edema, no difference in urine output was seen over 48 h in a burn and smoke inhalation sheep model [135]. However, it is suggested that fluid resuscitation alone cannot improve AKI once established. Thus, other approaches are necessary.

Animal models have also been used to improve upon other hospital-based treatments. Some of these currently implemented treatments include normobaric hyperoxia treatment to enhance oxygen delivery [62]. HS rats (MAP maintained at 30 ± 5 mm Hg) placed in a 1 atm/100% oxygen chamber had reduced numbers of necrotized tubular cells and lower serum creatinine levels when compared with HS mice placed in a 1 atm/20% oxygen chamber. The efficacy of these types of treatments should be investigated further using animal models.

The molecular mechanisms in response to HS or burn-induced AKI gained with animal models also allow investigators to identify and screen novel treatments for preventing or treating AKI. A quintessential example of this was recently published in Scientific Reports [136]. In this study, melatonin was shown to reverse burn-induced: increases in serum creatinine and BUN; increases in renal MDA, IL-1 β , TNF, ICAM-1, Bax, caspase 3, and TUNEL staining; and decreases in renal GSH, SOD, and bcl-2. However, the treatment in this case (i.e., melatonin) is not the novel aspect of the study and, as mentioned earlier, single therapies may not be sufficient for preventing HS- or burn-induced AKI. What this study did identify, however, is the crucial role of sirtuin-1 and its deacetylase activity on p53, p65, and FOXO1. This now identifies other potential pathways that can be leveraged for exerting beneficial effects on apoptosis, inflammation, and oxidative stress.

Due to the multifactorial nature of AKI-induction after trauma (either HS or burn), either a cocktail of pharmacotherapies, or perhaps a biologic therapy that exerts pleiotropic effects may be the most beneficial. For example, stem cell therapy has been touted for some time in a variety of diseases including burn and sepsis [137]. Animal models have examined this possibility in burn-induced AKI as well. One study utilized umbilical-cord derived mesenchymal stem cells (MSCs) in a 20% TBSA scald rat over the course of weeks [138]. Although untreated animals displayed 100% mortality in this study, MSC treatment reduced infiltration of inflammatory cells, normalized serum creatinine levels and improved survival. While baseline creatinine levels were not reported, these authors did show that apoptosis in the kidney was also reduced with MSC treatment. While the mechanisms responsible were not elucidated, it appears that anti-inflammatory properties of MSCs may be involved. Promising therapies such as MSCs should be studied further for their potential use.

Use of antioxidants as a protective measure against traumatic injury has also received much attention. As mentioned previously, the excess production of ROS and RNS generated from HS can be scavenged by various antioxidant defense systems thereby minimizing the impact of the inflammatory cascade. Crocetin, an antioxidant of the carotenoid family reduced iNOS expression and subsequent NO production in HS rats [58]. Crocetin prevented the HS-induced MDA spike and alleviated AKI as demonstrated by reduced creatinine. However authors did not report on Akt signaling mechanisms [58]. Additionally, the antioxidant activity of *Ginkgo biloba* extract reduced serum TNF- α , MPO activity, and creatinine levels in burned rats [71] and in unilaterally nephrectomized rats subjected to renal pedicle occlusion and reperfusion [139]. Others have targeted the mitochondria in an effort to prevent apoptosis. For example, cyclosporine A (CsA), a molecule that inhibits the opening of MPTP has shown benefit after HS. Polytrauma including HS + femur fracture in rats administered 5 mg/kg CsA had 56%

survival rate compared with 25% in rats who only received lactated Ringer's (LR) solution [60]. However, much remains to learn regarding the most effective antioxidants and the timing of treatment.

Other researchers have successfully targeted hormones and their functions in an effort to enhance the repair process. One of the most studied is erythropoietin (Fig. 2), which is a HIF-1 induced hormone that maintains survival of erythroid progenitor cells. Rats treated with erythropoietin (EPO) following HS but before resuscitation, had lower creatinine and urea nitrogen serum levels which may have partially contributed to decreased caspase activity [123]. Similarly, erythropoietin injected in rats 5 min prior to burn reduced caspase 3 activity, serum levels of TNF- α , IL-6, and IL-1 β and creatinine [122]. It should be noted that EPO administered in this study was prior to injury, which is not applicable to the clinical treatment of traumatic injury. In this regard, it may not be surprising that resuscitation requirements and mortality were not changed in clinical trials of rhEPO administration to burn patients [140], although AKI was not addressed.

8. Conclusion

No review is completely comprehensive, and the review presented here has focused on the development of AKI in relation to hemorrhagic shock and burn trauma. While there may be lessons learned from other models such as reports that include renal artery clamping or chemical-induced AKI, these studies should be interpreted with caution. Moreover, this review has focused on metabolic and immunological aspects of AKI development after traumatic injury that have been derived from HS and burn trauma animal models. While a number of hemorrhage or burn studies discussed focused on other (or multiple) organs, only a few were focused on renal dysfunction. Nevertheless, examining these studies identified some key molecular mechanisms that implicated inflammation and metabolic derangements in the disease progression including oxidant and nitrosative stress markers, cytokines, toll-like receptors, PAMPs, DAMPs, and complement. In addition, there is much interest in the role of mitochondrial damage and apoptosis in development and progression of AKI.

Considering the weaknesses in the current state of the literature discussed above, more research needs to be performed in a methodical fashion. For example, in our review we presented evidence for the role of p38MAPK in AKI, but additional mechanistic studies are needed to distinguish between true AKI and the natural physiologic response of the kidney to HS or burn injury that would not progress to chronic kidney injury. Rodent models, with a specific emphasis on conditional knockout models, as well as cell culture studies could be utilized as a screening tool for identifying both mechanistic pathways and therapeutic targets. Such knockout models could focus on specific pathways in the kidney (and even specific regions within the kidney) to examine which aspects of inflammation, etc. are essential to the development of AKI. An example could be the previously mentioned p38 pathway, which has an extensive downstream signaling network. Also, upstream p38 inhibition has been attempted in rheumatoid arthritis, but was discontinued largely due to liver toxicity. However, this might not exclude trauma from its potential therapeutic repertoire due to the acute nature of burn or HS.

If promising targets have been identified, large animal models could then be used for a second stage analysis of efficacy in different disease states. Large animals such as swine, are not only relatively similarly sized to humans, with similar biochemical values (i.e., creatinine), their response to trauma is also more akin to that seen clinically. Of note we have recently developed a swine burn model where indices of AKI have been observed by 2 h after injury [41]. We are planning to utilize this model in the future to investigate various treatment strategies to include the use of antioxidants, mitochondrial protective agents, complement inhibitors and stem cells, mentioned in this review.

In addition, the limitation of the various approaches to identify AKI is being supplanted by incorporation of multiple aspects of kidney

dysfunction to include KDIGO criteria. The result is progress towards improving translatability of animal studies to the clinical setting to improve treatment strategies for trauma-induced AKI. Certainly further research is necessary to fully understand mechanisms of renal injury induced by trauma. Overall, future strategies that incorporate multiple treatments, including biologics and cell therapies with pleiotropic effects, will undoubtedly be identified for the prevention and treatment of trauma-induced AKI.

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