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AWARD NUMBER: W81XWH-16-2-0041

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

PRINCIPAL INVESTIGATOR: David Burmeister

RECIPIENT: The Geneva Foundation

REPORT DATE: Oct 2019

TYPE OF REPORT: ANNUAL

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
Oct 2019	Annual	09/30/2018-09/29/2019

4. TITLE AND SUBTITLE
Resuscitation Strategies for Burn Injuries Sustained in Austere Environments to Improve Renal Perfusion and Function

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-2-0041

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dr. David Burmeister

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
US Army MRMC
Ft Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

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.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE	Unclassified	11	USAMRMC
Unclassified	Unclassified	Unclassified			19b. TELEPHONE NUMBER *(include area code)*

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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 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.

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

ACCOMPLISHMENTS:

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 will 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 the 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. 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 also reported the results of the CT-enhanced angiographies. This data has also been used as a baseline comparator for IV fluid experiments described 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. This information has recently been accepted for publication in the Journal of Surgical Research.

In terms of inflammatory cells, we have also has an accepted manuscript in Burns, delineating the state of splenic and circulating immune cells. Specifically, 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. We have also published on the adrenal response in this model, delineated in a manuscript using the adrenal tissues of these animals in the Journal of Burn Care and Research (2018 Aug 17; 39(5):652-660). We showed that IV fluids affect adrenal integrity, which is related to this objective, but not explicitly stated in the original SOW.

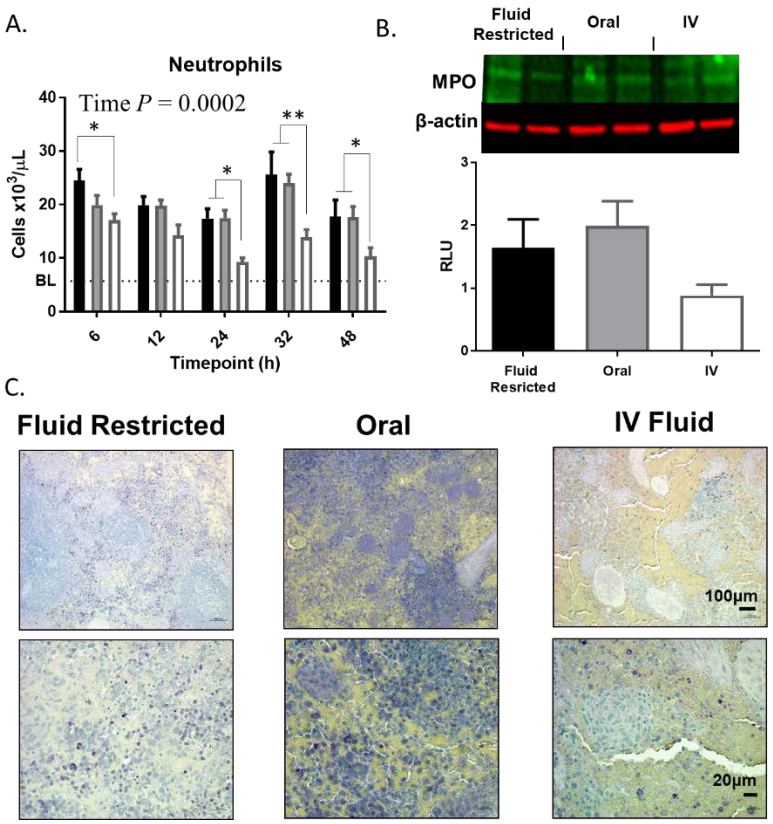

Figure 1. Expression of neutrophils in circulation and in the spleen of burned animals fluid restricted, or administered Oral or IV fluids. A, Plasma levels of neutrophils at baseline (BL) and 6, 12, 24, 32, and 48 h following burn. B, Representative western blot and expression of myeloperoxidase (MPO) in splenic lysates 48 h following burn. No significant differences were detected between treatments. C, Histological representation of MPO stain in splenic sections, scale bar 100μm in top panels and 20μm on bottom panels. Data displayed as mean ± SEM, n = 6; *P < 0.05 **P < 0.001 difference.

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 USAISRs 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. Additionally, the knowledge products generated form this objective has inspired an IRB-submission examining the feasibility of enteral fluids within the USAISR burn center. This cannot be understated that the **translatability** from this modest preclinical work has been realized.

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. In the past year, we have also published a knowledge report that IV fluids alter the gut microbiome in a dose

dependent manner (McIntyre, Shock 2019), and we have included this as an addendum. Tissue from these experiments are continuing to be analyzed along with those from Objective 2b.

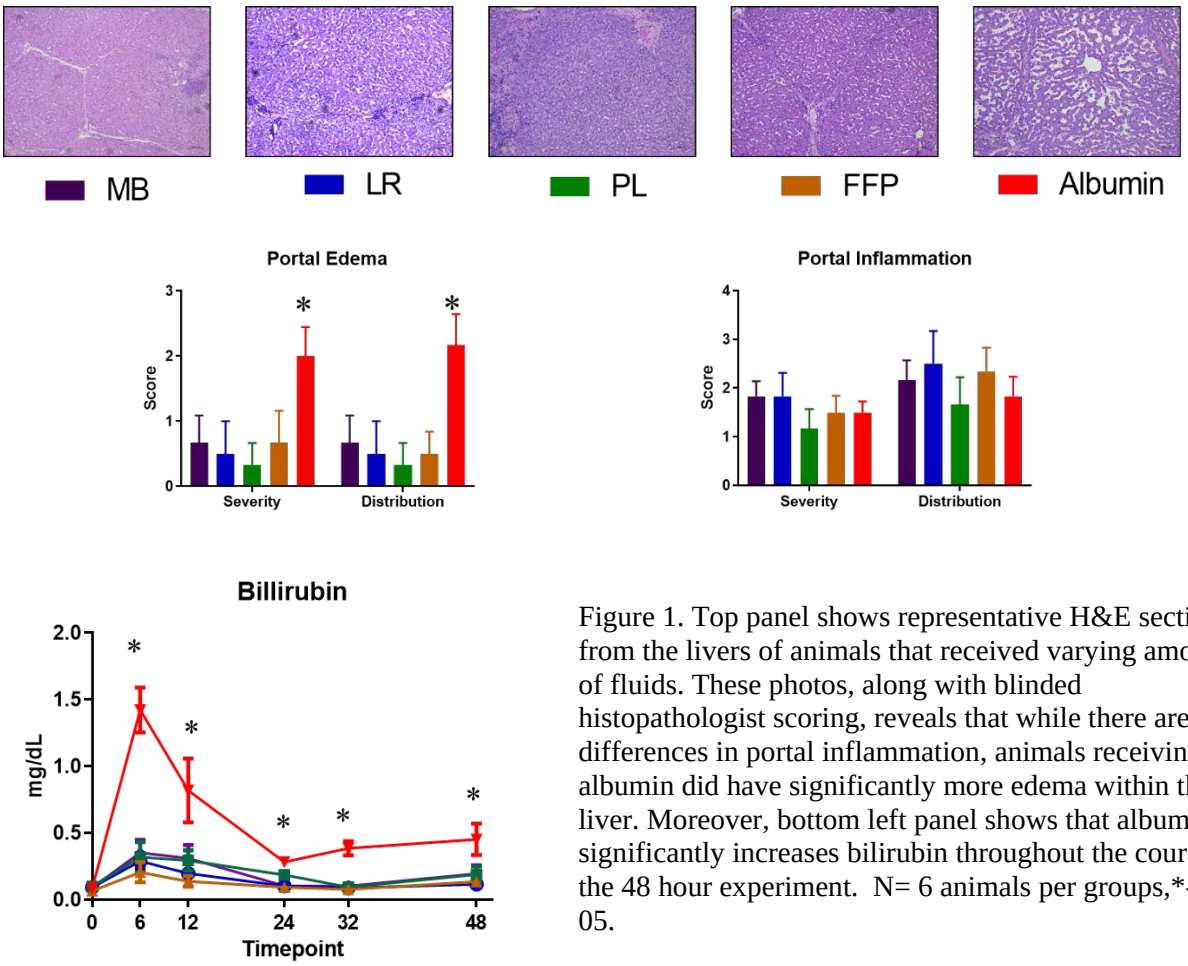

Figure 1. Top panel shows representative H&E sections from the livers of animals that received varying amounts of fluids. These photos, along with blinded histopathologist scoring, reveals that while there are no differences in portal inflammation, animals receiving albumin did have significantly more edema within the liver. Moreover, bottom left panel shows that albumin significantly increases bilirubin throughout the course of the 48 hour experiment. N= 6 animals per groups,*-P< 05.

Objective 2b: Animal experiments for this objective have been completed, and analysis of the biochemistry has been done with a manuscript in preparation on the livers from these animals. Specifically, Figure 2 shows that animals that received albumin have increased edema within the liver in terms of both severity and distribution. Moreover, animals receiving albumin had increased levels of bilirubin throughout the course of the experiment. These results have recently been presented at 2019 Military Health System Research Symposium, and are currently being prepared for manuscript submission.

2) Objectives 1 and 2: We have submitted a no-cost extension for this contract in order 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 recruited and hired as an assistant professor at University of Texas Medical Branch in Galveston, TX. This speaks to the success mentorship. As replacement fellow has just joined the lab. Mentorship 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- final preparation and submission of at least 2 distinct manuscripts related to the limited volume experiments (objectives 2a and 2b).

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. In fact, this project has inspired an IRB-submission which was completed this week. A prospective observational trial examining the safety and efficacy of enteral fluids is going to be funded with core USAISR funds.

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). Additionally, burn providers were briefed on results from this study in a staff development day. Bedside nurses are now on board with supporting a clinical study, which has been submitted. The knowledge products and translatability of the information gathered with this proposal has been a raging success.

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

No real issues to report since year 1. However, the shear amount of tissues and plasmas generated from this protocol have taken longer to process than was initially planned.

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.

1. Gomez BI, Harrington BK, Chao T, Chung KK, Dubick MA, Boggs NA, Burmeister DM. Impact of Oral Resuscitation on Circulating and Splenic Leukocytes After Burns. Accepted, Burns.
2. Chao T, Gomez BI, Heard TC, Dubick MA, Burmeister DM. Increased oxidative phosphorylation in lymphocytes does not atone for 1 decreased cell numbers after burn injury. Accepted, Innate Immunity.
3. McIntyre MK, Winkler CJ, Gomez BI, Lapierre JP, Little JS, Dubick MA, Nicholson SE, Burmeister DM. The Effect of Burn Resuscitation Volumes on the Gut Microbiome and Intestinal Absorption in a Swine Model. Shock. 2019 Oct 16. doi: 10.1097/SHK.0000000000001462, published, yes.
4. Burmeister DM, Little JS, Gomez, BI, Gurney J, Chao, T, Cancio, L, Kramer GK, Dubick MA. Operational Advantages of Enteral Resuscitation Following Burn Injury in Resource-Poor Environments: Palatability of Commercially Available Solutions. J Spec Oper Med. 2019 Fall;19(3):76-81, published, yes.
5. Chao T, Gomez BI, Heard TC, Smith BW, Dubick MA, Burmeister DM. Burn-Induced Reductions in Mitochondrial Abundance and Efficiency are More Pronounced with Small Volumes of Colloids in Swine. Am J Physiol Cell Physiol. 2019 Sep 18. doi: 10.1152/ajpcell.00224.2019, published, yes.
6. 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.
7. 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.

8. 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.

Selected abstracts that have been part of official publications, and/or presented as oral or posted presentations at national scientific and military meetings.

1. Belinda I. Gómez, Joshua. S. Little, Tiffany C. Heard, Michael A. Dubick and David M. Burmeister. Limited Volume Resuscitation with 5% Albumin Exacerbates Liver Injury in a 40% TBSA Swine Burn Model. Military Health System Research Symposium 2019 (Kissimmee, FL).
2. Little, JS, Gomez, BI, Gurney, J, Cancio, LC, Kramer, GC, Dubick, MA, Burmeister, DM. Palatability of Commercially Available Rehydration Solutions: Preferences of Active Duty
3. Service Members. Military Health System Research Symposium 2019 (Kissimmee, FL)
4. Matthew McIntyre, Charlotte J. Winkler, Belinda I. Gómez, Jean-Paul Lapierre, Joshua S. Little, Michael A. Dubick, Susannah Nicholson, David M. Burmeister. Intravenous Resuscitation Attenuates Gut Microbiome and Intestinal Changes after 40% TBSA Burn Injury in Swine. American Burn Association 2019 (Las Vegas, NV).
5. Burmeister, OM, Gomez, Bl, Chao, T, Gurney, JM, Kramer, G, Dubick, MA. The Operational Advantages of Utilizing Enteral Resuscitation for Severe Burn Injury in Prolonged Field Care Scenarios. Special Operations Medical Assembly, May 2018 (Charlotte, NC).
6. McIntyre MK, Winkler CJ, Gomez BI, Chao T, Little JS, Nicholson S, Dubick MA, Burmeister DM. (2018) Lactated Ringers Attenuates Gut Microbiome and Intestinal Changes after 40% TBSA Burn Injury in Swine. Oral Presentation at the 15th Annual Louis R.M. DelGuercio, MD Distinguished Visiting Professorship & Research Day. Valhalla, NY. December 19, 2018.
7. Tony Chao, Grace CY Chu, Belinda I. Gomez, Shanmugasundaram Natesan, Robert J. Christy, Michael A. Dubick, David M. Burmeister. Increased Mitochondrial Respiration and ROS Production from Adipose Derived Stem Cells is Passage Dependent. RegenMed San Antonio 2019.
8. 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.
9. 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.
10. 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.
11. 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.
12. 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.
13. 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.
14. 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.
15. 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.

16. 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.
17. 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**
  Enteral Fluid delivery standard operating procedure.

- **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:	12
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:	10
Contribution to Project:	Dr. Gomez assisted with animal procedures and processed blood/tissue samples.

Name:	Tiffany Heard
Project Role:	Research Lab Technician III
Nearest person month worked:	7.0
Contribution to Project:	Tiffany is learning assays that examine mitochondria function.

Name:	Joshua Little
Project Role:	Private First Class
Nearest person month worked:	6

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 charts: Attached

APPENDICES

1-136 R00 Oral Rehydration Solution (ORS) CPG.pdf
McIntyre_Shock.pdf
Burmeister_JSOM.pdf
Chao_AJPCell.pdf
Gomez_JBCR.pdf
Gomez_PLoS1.pdf
Burmeister_BBA.pdf

Title: Use of Oral Rehydration Solution (ORS) During Initial Management of Adult Burn Patients				No:	1-136
				Revision:	00
Type:	CPG	Task Area/Dept:	Burn Center	Page:	1 of 5

SIGNATURES AND DATES

Author:	Signature: On File	Date
	Name: Title:	
Changed By:	Signature: HOUSETON.KAREN.MARY. Digitally signed by HOUSETON.KAREN.MARY. Date: 2018.12.21 09:41:25 -06'00'	Date
	Name: Karen Houseton Title: CTR G2S Corp., Medical Device Regulatory Specialist and Document Control Manager	
Review:	Signature: FOR MS. TANYA LUCKADO	Date 21 DEC 2018
	Name: Tanya R. Luckado, MA, BSN, RN, CCRN Title: Burn Program Manager	
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	Name: Leopoldo C. Cancio, MD, FACS, FCCM, COL(ret), MC, USA Title: Director, Burn Center	
QA Approval:	Signature: N/A	Date
	Name: Title:	

Title: Use of Oral Rehydration Solution (ORS) During Initial Management of Adult Burn Patients				No:	1-136
				Revision:	00
Type:	CPG	Task Area/Dept:	Burn Center	Page:	2 of 5

1.0 PURPOSE

To establish a process for the administration of enteral resuscitation fluids in adult burn patients.

2.0 OVERVIEW

Patients with burns >20% TBSA require fluid resuscitation to address hypovolemia that develops secondary to capillary leak and increased insensible losses. Intravenous fluids (IVF) have been the mainstay of volume resuscitation in these patients. Enteral resuscitation has been found to be safe[1] and to reduce IVF requirements.[2] Reduction in IVF requirements may help prevent the co-morbidities associated with volume overload.

3.0 REFERENCES

All references are the most current version/revision unless otherwise specified.

Reference No.	Title
1	Milner SM, Greenough WB, 3rd, Asuku ME, Feldman M, Makam R, Noppenberger D, et al. From cholera to burns: a role for oral rehydration therapy. J Health Popul Nutr. 2011;29(6):648-51
2	Moghazy AM, Adly OA, Elbadawy MA, Hashem RE. Evaluation of WHO oral rehydration solution (ORS) and salt tablets in resuscitating adult patients with burns covering more than 15% of total body surface area (TBSA). Ann Burns Fire Disasters. 2016;29(1):43-7

4.0 ADDITIONAL READING

All references are the most current version/revision unless otherwise specified.

Title
SALINE solution in treatment of burn shock. Public Health Rep. 1950;65(41):1317-20
Baker BL, Powell D, Riesberg J, Keenan S. Prolonged Field Care Working Group Fluid Therapy Recommendations. J Spec Oper Med. 2016;16(1):112-7
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Cancio LC, Sheridan RL, Dent R, Hjalmarson SG, Gardner E, Matherly AF, et al. Guidelines for Burn Care Under Austere Conditions: Special Etiologies: Blast, Radiation, and Chemical Injuries. J Burn Care Res. 2016
Ivy ME, Possenti PP, Kepros J, Atweh NA, D'Aiuto M, Palmer J, et al. Abdominal compartment syndrome in patients with burns. J Burn Care Rehabil. 1999;20(5):351-3
Jeng J, Gibran N, Peck M. Burn care in disaster and other austere settings. The Surgical clinics of North America. 2014;94(4):893-907
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Michell MW, Oliveira HM, Kinsky MP, Vaid SU, Herndon DN, Kramer GC. Enteral resuscitation of burn shock using World Health Organization oral rehydration solution: a potential solution for mass casualty care. J Burn Care Res. 2006;27(6):819-25
Saffle JR. Fluid Creep and Over-resuscitation. Crit Care Clin. 2016;32(4):587-98. doi: 10.1016/j.ccc.2016.06.007
Vyas KS, Wong LK. Oral rehydration solutions for burn management in the field and underdeveloped regions: a review. Int J Burns Trauma. 2013;3(3):130-6

5.0 RECORDS

All references are the most current revision unless otherwise specified.

Identifier	Title
N/A	Burn Navigator entries

6.0 ABBREVIATIONS, ACRONYMS AND TERMS

Identifier	Description
GI	Gastrointestinal
IVF	Intravenous Fluid
NGT	Nasogastric Tube
ORG	Orogastric Tube
ORS	Oral Rehydration Solution
TBSA	Total Body Surface Area
WHO	World Health Organization

7.0 MATERIALS/EQUIPMENT

- ORS
- Burn Navigator

8.0 SAFETY INFORMATION

See Exclusions.

9.0 RESPONSIBILITIES

As defined throughout this procedure.

10.0 PROCEDURE

ORS Administration

10.1 Per physician discretion, oral rehydration solution (ORS) can be administered via a nasogastric tube (NGT) or orogastric tube (OGT) as part of the initial fluid resuscitation (within the first 24 hours).

10.2 The World Health Organization recommends pre-packaged ORS that contains 2.6 g sodium chloride, 1.5 g potassium chloride, 2.9 g trisodium citrate, and 13.5 g glucose per liter. ORS via the NGT or OGT is initiated with the head of bed elevated by 30-45 degrees, after the initial shower is completed and after the placement of the NGT or OGT is confirmed by x-ray. The gastric motility agent naloxone (2-4 mg Narcan q4h) is given enterally upon initiation of ORS to aid in GI motility. Gastric residuals are checked to ensure they are less than 300 cc prior to initiation of ORS. If residuals are over 300 cc on initial check, dispose of the gastric contents and check residuals again in 1-2 hours to see if initiation of ORS is warranted.

10.3 The Burn Center nursing staff mixes one ORS packet in 1 L of sterile water according to the package directions. When reconstituted, the date and time shall be labeled accordingly. The solution can be used up to 24 hours after reconstitution but then must be discarded.

10.4 Reconstituted ORS is placed in the enteral bag (rather than the flush bag) for the eKangaroo pump. When enteral nutrition is initiated during ORS use, the enteral nutrition formula is placed in the flush bag and started at 20 cc flushes each hour, increasing per usual practice, but as boluses rather than as a continuous drip until ORS fluid resuscitation is completed.

10.5 ORS is initiated through the NGT or OGT at 200 cc/hour and is increased by 100 cc/hour up to 400 cc/hour.

10.6 Gastric residuals are initially checked hourly, and then extended to every other hour by physician order based on tolerance (i.e., residuals <300 cc). ORS administration, if stopped, can be reinitiated in 2 hours at 200 cc/hour. If low-volume vomiting occurs, ORS can be re-initiated (assuming low residuals) in as early as one hour; restart at 200 cc and advance as tolerated to goal.

10.7 The usual computerized decision support (i.e., Burn Navigator) for fluid resuscitation is used and the IVF rate is adjusted in the usual fashion, as the fluid absorption via the GI tract can be variable. Additionally, this decision support system will be used to document enteral fluids.

10.8 ORS administration will stop when the decision support system is discontinued.

11.0 EXCLUSIONS

DO NOT USE ORS in any of the following situations:

- GI tract not appropriate for enteral fluid resuscitation (i.e., recent gastric bypass surgery, which could cause dumping, or GI tract not in continuity).
- Significant vasopressor use, defined as norepinephrine > 5 mcg/min (with or without vasopressin).
- Burn size ≥50% TBSA
- Admission ≥24 hours after injury
- Age ≥65 years old
- Baux score ≥100

12.0 EXPECTED OUTCOMES

- Safe administration of ORS: gastric residuals <400 cc, no aspiration
- Correction of burn shock with improvements in base deficit, lactate, vasopressor use
- ORS may result in lower IVF requirements during the initial fluid resuscitation
- ORS may decrease total resuscitation volumes and related complications of edema

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13.0 APPENDICES

Identifier	Title
N/A	N/A

14.0 REVISION HISTORY

Rev	Effective Date	Description of Change	Changed By:
00	28Dec2018	New	N/A

Shock, Publish Ahead of Print

DOI : 10.1097/SHK.0000000000001462

The Effect of Burn Resuscitation Volumes on the Gut Microbiome in a Swine Model

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Funded by US Army Medical Research Development Command W81XWH-16-2-0041.

Running head: Burn Resuscitation Influences Gut Microbiome

Abstract

Introduction: While recent reports underscore the significance of the gut microbiome (GM) in health and disease, its importance in burn outcomes remains unclear. Moreover, aggressive intravenous (IV) fluid resuscitation of patients may alter intestinal flora. Herein, we describe GM changes following a large burn in swine randomized to different volumes of IV Lactated Ringers' (LR).

Methods: Anesthetized Yorkshire swine sustained 40% Total body surface area full-thickness burns and were randomized to different volumes of IV LR: none (n=3), 15mL/kg/day (Low; n=6), or 80mL/kg/day (High; n=6). At baseline and days 1 and 2, fecal swabs were collected for 16s rDNA sequencing. Proximal jejunum was collected immediately after euthanasia (day 2) for western blot, histopathology, and cytokine analyses.

Results: Burns produced significant shifts in β-diversity and non-significant reductions in α-diversity that did not recover regardless of treatment group. Burn-induced increases in Proteobacteria and decreases in Firmicutes were attenuated by IV fluids in a dose-dependent manner, and also correlated with α-diversity. IV fluids caused a dose-dependent increase in *Bacteroides* and prevented a transient increase in the opportunistic pathogen *Haemophilus parainfluenzae*. While high volumes of IV fluids increased intestinal Hsp70 levels (p=0.0464), they reduced SGLT1 (p=0.0213) and caspase3 (p=0.0139) levels. IV fluids elicited a non-specific cytokine response however Bacteroidetes levels correlated with intestinal IL18 levels (p=0.0166, R^2=0.4201).

Conclusions: We present the first report on the gut microbiome in a porcine burn model, and present data to suggest that IV fluids may influence GM and gut functional proteins following a burn. Overall, burn injury results GM diversity shifts, which may expose diagnostic and/or therapeutic targets to improve outcomes.

Keywords; burn, resuscitation, gut, microbiome, intestine, diversity, inflammation, swine

Introduction

Extensive burn injury results in significant pathophysiologic stress accompanied by marked systemic inflammation that may lead to multi-organ failure (1,2). As such, burns are associated with significant morbidity and mortality leading to approximately 11 million hospitalizations and 300,000 deaths worldwide annually (3). The management of burn patients has evolved to include aggressive fluid resuscitation, early debridement/grafting, and enteral feeding that have collectively improved outcomes (4). Still, the burn patient is highly susceptible to infectious complications such as sepsis and pneumonia, which have deleterious consequences on outcomes and costs. As a correlative, it is thought that sepsis development following a burn injury may be due to increases in intestinal permeability leading to microbe leakage (5–8). Despite this, there remains a paucity of evidence on how to manage compromised gut integrity following severe burns.

Recent advances in sequencing technology have been leveraged to elucidate the complex ecosystem termed the microbiome that lives in and on our bodies. While several studies have examined burn wound outcomes and the skin microbiome (9,10), relatively few studies have investigated the GM following large burn injury. GM shifts are described by observing changes in specific taxonomic levels and by using α-diversity (a measure of species distribution within a sample) and β-diversity (a comparison of microbial composition between groups) metrices. Thus far, one rodent study has shown burn-induced shifts in the GM may allow for proliferation of pathogenic organisms (11). The implications of these changes are still murky but it is thought a pathobiome may contribute to later sepsis development or other forms of multi-organ dysfunction.

While massive vascular leakage and fluid shifts post-burn have been countered with aggressive intravenous (IV) fluid resuscitation, there remains variability in resuscitation

strategies and volumes within the burn community (12). Moreover, large amounts of IV fluids can lead to clinical sequelae such as compartment syndromes or acute respiratory distress syndrome. The extent to which fluid levels effect the GM is largely unstudied. To this end, the current study investigates the effect of different resuscitation volumes on the GM and proteins critical for intestinal absorption utilizing an established porcine 40% TBSA burn model (13). We hypothesized that a shift in the GM following burn injury would be modulated by varying fluid resuscitation volumes.

Materials and Methods

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. Seventeen three-month-old sexually immature female Yorkshire (*Sus Scrofa*) pigs (weighing 40.9 ± 1.1 kg) were included in this study, which represented a substudy of previously published work (14). 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 consistent commercial laboratory porcine formulated pelleted diet (Laboratory Mini-Pig Grower Diet, Cat# 5081, LabDiet, Richmond, IN). Animals were randomly allocated to one of three treatments prior to thermal injury: restricted fluid group (None; n=5), or IV lactated Ringer's solution at 15mL/kg/day (Low; n=6), or IV 80mL/kg/day (High; n=6).

Thermal Injury and Follow-up

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. Creation of 40% TBSA burn full-thickness contact burns was performed as previously described (13). Briefly, animals were anesthetized with an intramuscular injection of tiletamine-zolazepam (Telazol, 6 mg/kg), intubated, and placed on a ventilator with an initial tidal volume at 10mL/kg, a peak inspiratory pressure of 20 cm H_2O, and respiratory rate of 8 to 10 breaths/min. The ventilator was adjusted to achieve an end-tidal PCO_2 of 40±5 mm Hg. Animals were maintained on 1% to 3% isoflurane, balance O_2 anesthesia. Hair was removed from the dorsum, flanks, and legs using clippers and razors with shaving cream. Intravenous access was created using standard cut-down procedures 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±0.2ºC by a temperature controller. Heated probes were placed against the skin for 30s to produce full-thickness burn injuries. This procedure was repeated until 40% of the TBSA was burned.

Burn wounds and post-injury follow up

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 from anesthesia and kept in a metabolic cage (15) (dimensions 41'L x 16' W 44' H) for monitoring IV fluid intake as previously described. Feed was given once animals were awake and standing independently. Because of the unfamiliarity with the smaller metabolic cage, 5

animals showed signs of distress (e.g., vocalization, jumping) and were administered intramuscular midazolam (0.1±0.25 mg/kg) for light sedation. Animals were fed the same formulated chow post-burn, and there was no apparent difference in the slightly reduced appetites across groups. 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). At this time rectal swabs were obtained for sequencing analysis and placed at -80°C until all samples were collected. Upon euthanasia at 48 hours, intestine samples from the proximal jejunum were immediately preserved in 10% neutral buffered formalin, embedded in paraffin wax, and sectioned into 4-μm slices.

CT Analysis

At baseline and 48 hours, superior mesenteric artery cross-sectional area was visualized with contrast-enhanced computerized tomography (CT) scans. Under anesthesia, 40 mL of Iopamidol (755 mg/mL) was injected into an ear vein catheter. Obtained CT images were transferred to an independent Vitrea 3D workstation (Vitrea Version 6.7.4; Vital Image Inc., Minnetonka, MN) for measurement. The Vitrea Advanced 3D Vascular: Runoff CT protocol was used for analysis of the superior mesenteric artery, and the Vessel Probing tool was utilized to probe the superior mesenteric artery 0.5mm to 1.0mm in the same anatomic plane as the first junction of the celiac artery.

Histology

Proximal jejunum samples were preserved in 10% neutral buffered formalin for a minimum of 48 hours, embedded in paraffin wax, and sectioned into 4 μm slices. Following deparaffinization samples were stained with wheat germ agglutinin and counterstained with phalloidin and DAPI. Additionally, TUNEL staining was performed (C10617, ThermoFisher

Scientific, Waltham, MA) according to the manufacturer's instructions. Intestine slices were imaged using an (Carl Zeiss, Thornwood, NY) and put through automated quantification of colors with ImageJ software version 1.51d (Bethesda, MD). Ensuing images were separated into red, green, and blue channels for quantification of channel intensities.

Protein Analysis

To quantify total Caspase, SGLT1, AQP1, HSP70, and beta-actin within the intestines Western blots were performed by separating 50µg on 20% SDS-PAGE gels, and a semi-dry transfer to nitrocellulose membranes. Secondary antibodies (1:20,000) IRDye 800CW or IRDye 680RD (LI-COR) were applied for compatibility with the Odyssey infrared imaging system (LI-COR) and signal intensity was normalized to beta actin. For detection of tissue cytokines, intestines were lysed in Milliplex lysis buffer (EMD Millipore, Billerica, MA) and a porcine-specific multiplex kit (Millipore; PCYTMG-23K13PX) was used according to the manufacturer's instructions.

Sequencing Analysis

Swabbing was performed using sterile cotton tipped applicators (ThermoFisher Scientific) and stored at -80°C until analysis. Fecal DNA was isolated with the QIAamp DNA Stool Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. All samples (except 2 with insufficient DNA yield in the none group) underwent 16S rRNA amplification, library creation, and sequencing using V1_V2 16S Illumina MiSeq 600 pipeline using the 27F/R338 primer pair was used (Forward: NAGAGTTTGATCMTGGCTCAG; Reverse: NGCTGCCTCCCGTAGGAGT) (16). Sequences were deposited in the NCBI Sequence Read Archive under the bioproject ID#PRJNA573749 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA573749).

Sequencing analysis was performed using QIIME2-2017.10 given its established high performance in microbiome analysis. (17) 5,043,315 sequences across all samples were identified after merging and demultiplexing. Sequence quality control, feature table construction, and chimera removal was performed using the DADA2 pipeline (18). A right truncation was performed at 200bp based on a median quality score value that was >30, and aligned representative sequences were subsequently determined using FastTree 2 (19). Sample depth for diversity measures was set at 42,100bp to retain 60.51% of reads and 100% of samples. Alpha (Shannon diversity, Observed Taxonomic Units (OTUs), Faith Diversity, and evenness) and beta (Jaccard, Bray-Curtis, weighted/ unweighted UniFrac) diversity measures were calculated, and visualized using the Emperor program. OTU classification was accomplished using the Greengenes 13_8 database. Taxa were then assigned using the trained classifier and differential abundance was determined.

Statistics

Two-way ANOVA analysis with Tukey's post-testing was performed at each taxonomic level to determine differences in diversity between groups. Western blot and cytokine data were analyzed using a 1-way analysis of variance method (ANOVA) assuming Gaussian distribution. To detect treatment differences Tukey's multiple comparison test was performed. PERMANOVA in QIIME2 was used for beta diversity measure analysis. Unless otherwise stated, values are represented as arithmetic mean ± SEM. Statistical analysis and figure generation was performed using Graphpad Prism 7.0e (Graphpad Software, San Diego, CA).

Results:

Sequencing using the Illumina pipeline identified 5,043,315 total sequences (average of 114,620/sample). Following quality control, and removal of chimeras, 17,052

representative sequences were used for phylogenic tree generation. Eighteen unique phyla were identified with Bacteroidetes (32.3%), Firmicutes (30.9%), Proteobacteria (15.2%), and Spirochaetes (3.2%) being the most prevalent. Forty unique genera were also found, with *Prevotella* (18.9%), *Sulfurimonas* (14.3%), *Treponema* (14.0%), and *Ruminococcus* (12.9%) representing the four most commonly observed. Phyla and genera distributions for individual animals are shown in **Supplemental Figure 1, Supplemental Digital Content 1, http://links.lww.com/SHK/A962**. While baseline variability existed, no differences in β-diversity measures unweighted and weighted UniFrac plots (p=0.781, p=0.379, respectively), or α-diversity measures Shannon diversity (p=0.86), evenness (p=0.80) faith phylogenic diversity (p=0.44), or observed OTUs (p=0.77) were seen between study groups at baseline (**Supplemental Figure 2, Supplemental Digital Content 2, http://links.lww.com/SHK/A963**).

Burn injury induced significant differences in several phyla which were differentially affected by IV fluids. Proteobacteria were markedly elevated at day 1, but most dramatically in the none group (15.2% v. 39.5%; p<0.0001) compared to baseline (**Figure 1A**). By day 2, Proteobacteria levels remained elevated in the low group (p=0.0062). Significant decreases in Firmicutes at day 1 in all groups were also most dramatic in the none group (31.4% v. 18.7%; p=0.0197) and became non-significant by day 2 in all groups (**Figure 1B-C**). Non-significant decreases in Bacteroidetes levels were observed on day 1 for the low and none groups that returned to baseline by day 2 (**Figure 1D**).

At all lower taxonomic levels, differences based on treatment group and study day were found (**Supplemental figure 3, Supplemental Digital Content 3, http://links.lww.com/SHK/A964**). Notably, the family *Enterobacteriaceae* was increased across all time points and treatment groups. At the genus level, a dose- and time- dependent increase in the *Bacteroides* genus was associated with increased fluid levels, with concurrent

decreases in *Prevotella*. At the species level, increases in *Haemophilus parainfluenzae* and decreases in *Prevotella copri* were transiently exacerbated by fluid restriction **(Supplemental Figure 3, Supplemental Digital Content 3, http://links.lww.com/SHK/A964)**.

For α-diversity, a drop in observed taxonomic units (OTU) became significant by day 2 for animals that received fluids (p<0.022) (**Figure 2A**). Similar decreases in Shannon (p=0.19), faith (p=0.31), and evenness (p=0.53) were not statistically significant (**Figure 2B-D**). Interestingly, day 2 Firmicutes (**Figure 2E**) levels positively correlated with, OTU (P=0.0009; r^2=0.58), Shannon diversity (p<0.0001; r^2=0.78), and Evenness (p<0.0001; r^2=0.73), while day 2 Proteobacteria (**Figure 2F**) inversely correlated with these (p=0.014; r^2=0.38, p=0.0045; r^2=0.48, p=0.0071; r^2=0.44).

Measures of β-diversity revealed a significant shift in the microbiome following burns regardless of IV fluids (**Figure 3A-D**) for all 4 β-diversity measures studied (Jaccard similarity, p=0.001, Bray-Curtis, p=0.002, unweighted UniFrac distance, p=0.023, and weighted UniFrac distance, p=0.012). Largely, post-hoc analysis showed differences between baseline and ensuing days, but no further shifts between days 1 and 2. To examine the potential confounding effect of midazolam administration, we compared the day 2 GM β-diversity between animals that received midazolam and those that did not, and found no significant effect of benzodiazepine administration on the GM in terms of either α- or β-diversity (**Supplemental Figure 4, Supplemental Digital Content 4, http://links.lww.com/SHK/A965**).

High levels of IV fluids led to a non-significant increase in goblet cell area and decrease in wet-to-dry ratio (**Supplemental Figure 5A-E, Supplemental Digital Content 5, http://links.lww.com/SHK/A966**). As a proxy for gut perfusion, CT analysis of the superior mesenteric artery cross sectional area (**Figure 4A-C)** revealed that burn injury reduced the

size of the mesenteric artery (p<0.0001), which was exacerbated in the none group, but not different between the low and high groups.

Labelling of apoptotic cells with TUNEL staining showed an increase in apoptotic cells within the epithelium and lamina propria of animals that did not receive IV fluids (**Figure 5A).** Moreover, quantitative Westerns revealed high levels of fluids reduced the amount of Caspase-3 (p<0.0139). Western blotting also revealed that high fluid levels were associated with decreased active transporter SGLT1 (p<0.0213) and non-significant decreases in the passive transporter AQP1 (p=0.0976). Lastly, IV fluids also increased HSP70 (p<0.0464) levels in a dose-dependent manner (**Figure 6**).

Cytokine analysis showed that low amounts of fluids significantly elevated IL-1α (p=0.03), and IL-12 (p=0.04) levels (**Supplemental Table 1, Supplemental Digital Content 6, http://links.lww.com/SHK/A967**) within the intestine. Interestingly, SGLT levels were linearly correlated with increased tissue levels of IL-8 (p=0.0441; $r^2 = 0.2964$) and IL-10 (p=0.0490; $r^2 = 0.2856$) (**Supplemental Figure 6A, Supplemental Digital Content 7, http://links.lww.com/SHK/A968**). Further analysis revealed that Bacteroides levels were positively correlated with tissue IL-18 (p=0.0166; $r^2 = 0.4201$) (**Supplemental Figure 6B, Supplemental Digital Content 7, http://links.lww.com/SHK/A968**). Inverse correlations between day 2 Faith diversity and anti-inflammatory cytokines IL-2 (p=0.034; r^2=0.35), IL-4 (p=0.039; $r^2 = 0.33$), and IL-10 (p=0.039; $r^2 = 0.33$) within the intestine (**Supplemental Figure 6C-E, Supplemental Digital Content 7, http://links.lww.com/SHK/A968**) were also found.

Discussion

Recent advances in sequencing technology have yielded a panoply of information on the microbiome in health and disease, yet the microbiome's effect on burn outcomes remains

unclear. Early and aggressive fluid resuscitation is a mainstay of burn management: however, there also remains a paucity of data on how this treatment strategy influences the GM. To examine these potential effects, we used 16S sequencing of the fecal microbiome and tissue protein analysis in an established porcine model of 40% TBSA thermal injury undergoing different fluid resuscitation strategies. To our knowledge, this is the first report characterizing the gut microbiome of swine following burn injury. Significant perturbations in the GM following injury were demonstrated, including a Proteobacteria -centric community with large shifts β-diversity. Fluids did mitigate burn induced changes in certain taxa including Proteobacteria phyla and *Bacteroides* genus.

Following trauma, it has been well established that the GM is altered in both humans (20–23) and animal models (24) however, the implications of these changes are unclear. Burns represent a unique subset of trauma patients due to systemic inflammation, and burn-induced pathophysiology extends to the airway (25), skin (10), and, importantly, gut (11,26) microbiomes. The acute post-burn phase, the ebb phase, is characterized by tissue hypoperfusion and ischemia which is partially allayed through aggressive fluid resuscitation. Wang *et al* illustrated the temporal effect of burn injury on the GM in patients, by showing a distinct microbiome population early (i.e., <1 month after injury (*Enterococcus* and *Escherichia*)) and late (*Bacteroides*) in the post-injury period. (27). We present a hyperacute spike in potentially pathogenic organisms (Proteobacteria) immediately following injury, and that high fluid volumes can expedite a reemergence of beneficial organisms such as the *Bacteroides* genus.

Understanding GM diversity has the potential to identify diagnostic or therapeutic interventions. Alpha diversity measures represent species diversity within each fecal swab, with indices such as Shannon index accounting for species diversity, evenness accounting for species balance, and Faith indices considering the extent of phylogenetic diversity. One

previous rodent model of 30%TBSA burn injury has showed that α-diversity is relatively unchanged in the short-term following burn injury (28). Unlike that study, we saw significant differences in OTUs, which we believe is dependent on the extent of-, and time after injury. They also mentioned qualitative perturbations to one β-diversity measure (differences in community composition amongst groups), which we confirmed with statistical PERMANOVA analyses. Rodent models have also been used to examine the effect of alcohol (29) and age (30). While these studies give valuable insight to burn-induced gut function, the advantage of using a porcine model instead of rodents lies in the similarities between pigs and humans in the cutaneous (31) and intestinal (32) microbiomes along with the structure and healing properties of the skin itself (33). Therefore, despite increased cost and logistical challenges, the advantage of using a porcine model for the study of burn injury lies in the similarities between human and pig physiology. As such, we believe that we are the first to describe the shift in the GM β-diversity in a porcine model following a large burn injury, which may provide the groundwork for future study into the effect of clinical interventions on the GM.

A long-standing hypothesis for sepsis involves increasing intestinal permeability following burn injury leading to the bacterial translocation (5–8), which may be related to a dysbiotic GM (11,34). For example, while *Bacteroides fragilis* (a common anaerobic commensal) rarely causes bacteremia, there are instances of anaerobic sepsis (35). Although the mucus-producing capabilities of *B. fragilis* are generally viewed as beneficial, it has been shown that toxins specific to this anaerobe can cause sepsis in both humans and animals (36,37). Whether this is a possibility in the indistinct onset of sepsis in burn injury is not clear. However, the *Bacteroides* genus is generally considered to be a beneficial enteric organism due to its effects on complex sugar breakdown and maintenance of energy requirements (38). Furthermore, given the role *Bacteroides* plays in complex sugar

breakdown and absorption, it is interesting high IV volumes increased this genus but decreased SGLT expression. Promoting an accelerated shift toward *Bacteroides* could potentially have widespread effects on burn outcomes due to the increased metabolic demand following injury.

Fluids reduced expression of both active and passive water transporters, which may represent a compensatory mechanism to counteract compromised blood flow as measured by mesenteric artery diameter. We also found decreased apoptosis with IV fluid use with increased heat shock protein in the high fluid group. While limited by a small sample size and moderate variability, these results present circumstantial evidence that instead of undergoing apoptosis, cells in the high fluid group are compensating with heat shock proteins. Prior work has also shown a relationship between apoptosis and HSP70. Specifically, Yuan et al, showed that treatment with sodium arsenite both increases HSP70 and decreases apoptosis indicating the possibility of a shared mechanism (39). We were surprised to find a lack of relationship between these markers and microbiome characteristics.

While the ebb phase of burn injury is mediated by a milieu of pro-inflammatory cytokines in circulation, herein intestinal cytokine expression was mixed, and the only difference due to fluids was higher IL-1α and IL-12 levels in the low fluid group only. An interesting study by Tadros *et al* found that directly administering IL-1α following a burn may be protective for mesenteric blood flow and intestinal permeability (40). In this context, we cannot rule out the possibility of over-resuscitation in the high group (41,42). The observation of a correlation between IL-18 and the phylum Bacteroidetes is an interesting one, and likely tied to the role of IL-18 in gut integrity, which is implicated in burns (43), and intestinal inflammasome activation (44). While it is enticing to speculate that a specific

bacteria within the Bacteroidetes phyla could be responsible for maintaining this balance, we were not able to identify a correlation at any further taxonomic level.

The pig GM shares many common species with humans, but does display differences. For example, while the *Treponema* genus identified in the pig GM (45) is not usually found in human digestive tracts, it has been shown in the guts of hunter-gatherers (46). This brings to light one key variable that dictates the flora of the gut: diet confounds interpretation of human GM studies (47), which can be controlled for in animal models. We found that burn injury induced opposing changes in the genera *Bacteroides* and *Prevotella*, which are thought of as products from diets rich in animal protein/fats and plant-based carbohydrates, respectively (48). Moreover, these two genera have distinct production of short chain fatty acids due to fiber utilization (49), and *Prevotella* may preferentially support weight loss (50). Resuscitation adjuncts that promote *Prevotella* species levels in the gut may be a worthwhile avenue of research.

The main limitation of this study lies in the lack of insight into location-specific changes in the microbiome, as small vs. large intestinal variation exists (32). Given that we used fecal swabs, our results inform effects of burns on GM of the colon. While this is in contrast to the histological and molecular analyses from the jejunum (and thus may explain the lack of correlations between GM and proteins), we feel it represents a more feasible target for clinical translation. The other limitation of our study lies in the limited number of animals used in this study which hampered statistical power. Moreover, the lack of non-burn shams in our study complicates the interpretation of data where pre-burn measures are not available (i.e., the molecular data). However, this also highlights the potential non-invasive diagnostic power of the GM, which could be used to study the independent effect of resuscitation on gut flora. Finally, given our data showing a significant effect of resuscitation strategy on superior

mesenteric artery diameter, further work is needed to determine whether resuscitation influences the GM directly or rather as a consequence of perfusion.

Conclusions

Early and aggressive fluid resuscitation is a hallmark of burn treatment. The utility of the Brooke versus the Parkland formulas is a hotly contested area. This study is the first to show that the GM is altered following a large burn injury in pigs and that the GM may be influenced by the resuscitation strategy used. We went on to show that varying fluid resuscitation strategies influence key functional proteins in the small intestine. Given that the GM is altered following a burn, further mechanistic study is needed to determine the GM effects on burn outcomes. Such future studies that manipulate the microbiome using, for example, fecal transplant or antibiotics could examine how volume resuscitation influences outcomes following burn injury in hopes of elucidating the critical role that the GM plays in burn physiology and resuscitation outcomes. Moreover, the interaction between the GM and burn outcomes remains elusive. However, future clinical trials of resuscitation strategies should include GM analysis.

Competing Interests

The authors declare no competing interests.

Authors' Contributions

DB, SN and MD conceived the study. MM and DB prepared the manuscript. BG, JL and DB performed the animal experiments. MM analyzed the sequencing data. CW, JL, and JPL performed histological, imaging, and Western Blot analyses. All authors read and approved the final manuscript.

Acknowledgements

The authors would like to thank the Veterinary Support Branch for their support.

The assertions and opinions contained herein are solely those of the authors and do not represent those of the United States Army or the Department of Defense.

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Figure 1. Changes in different phyla of the gut post-burn. (A) Percentages of the top 4 phyla found across time in all animals (Day 0, n=15) and with increasing amounts of fluids (Day 1, 2: n=3, 6, and 6 in None, low, and high, respectively). Significant changes in Proteobacteria (B), Firmicutes (C), and Bacteroidetes (D) were seen. *-p<0.05, **-p<0.01, ****-p<0.0001 as compared to Day 0 (dotted lines).

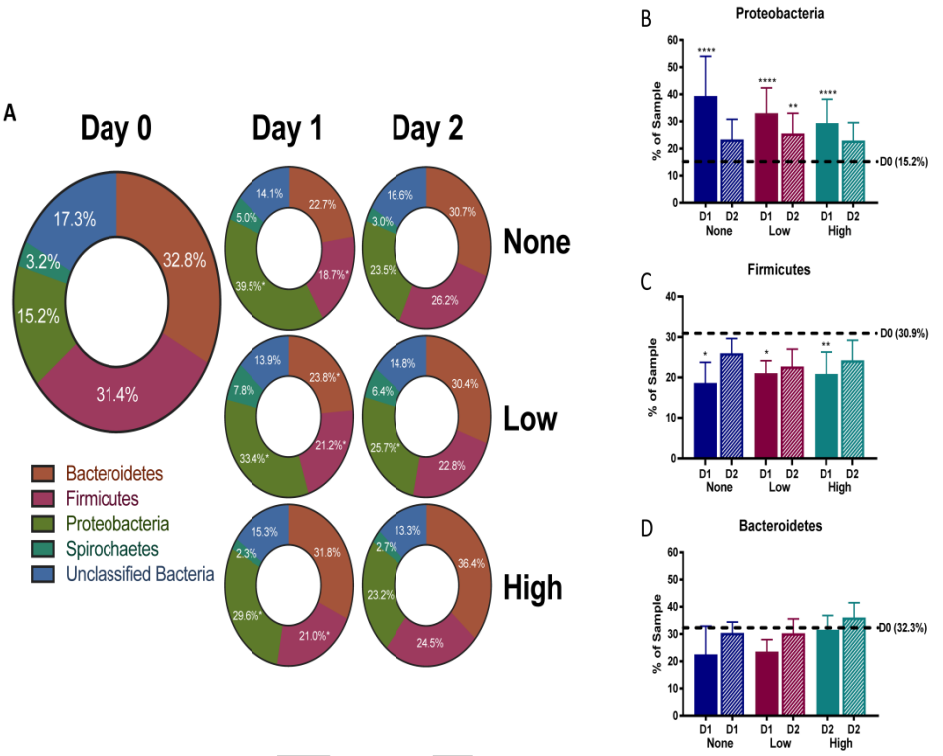


Figure 2. Alpha diversity measures across time. Significant differences in OTUs (A) were seen by Day 2 in animals that received IV fluids *=P<0.05. Slight decreases in Shannon Diversity (B), Evenness (C), and Evenness (D) were observed but did not reach statistical significance. Three measures of α diversity (OTUs, Shannon, and Evenness) positively correlated with the phyla Firmicutes (E) (Y=18.70*X + 239.6, Y = 0.1071*X + 5.278, Y = 0.007968*X + 0.6447, respectively) and negatively correlated with Proteobacteria (F) (Y = -9.786*X + 923.3, Y = -0.05412*X + 9.147, Y = -0.003998*X + 0.9319, respectively).

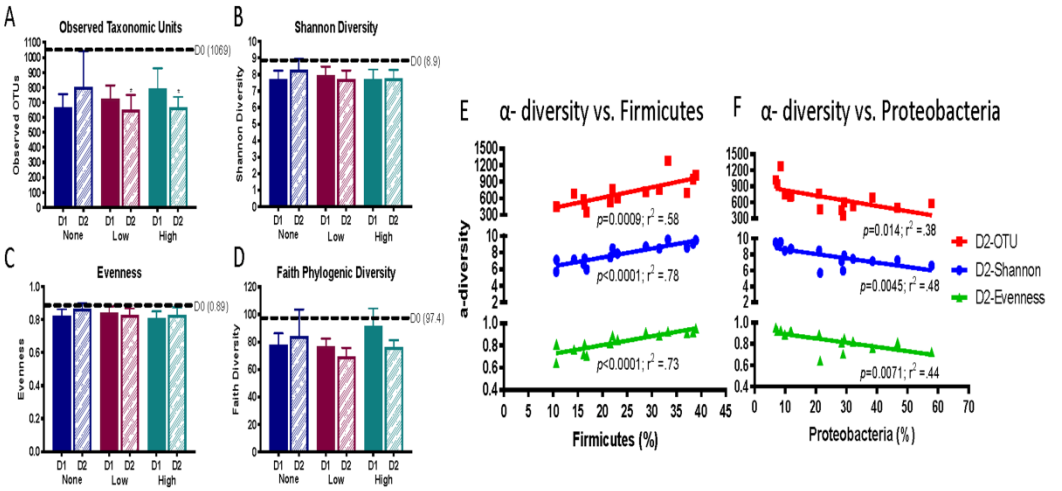


Figure 3. Shifts in Beta Diversity across Time. For β diversity, PERMANOVA analyses revealed a significant effect of time (but not IV fluids) for Jaccard similarity (A), Bray-Curtis (B), Unweighted (C) and Weighted Unifrac (D) analyses. Day0 = red dots, Day 1= Blue dots, Day 2 = yellow dots.

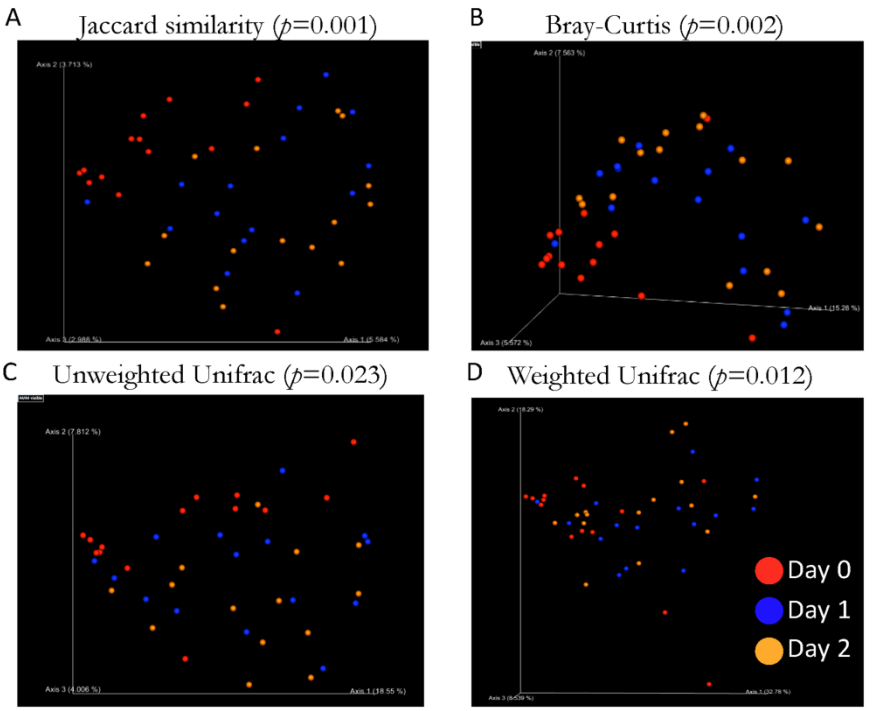


Figure 4. Gut perfusion an absorption as measure by CT. An example mesenteric artery measurement at day 0 is shown in (A), with day 2 pictures in each group shown in (B). The change in artery cross sectional area measurements is shown in (C), with significantly larger decreases in animals that received no IV fluid (****-p<0.0001, n=3, 6, and 6 in none, low, and high, respectively).

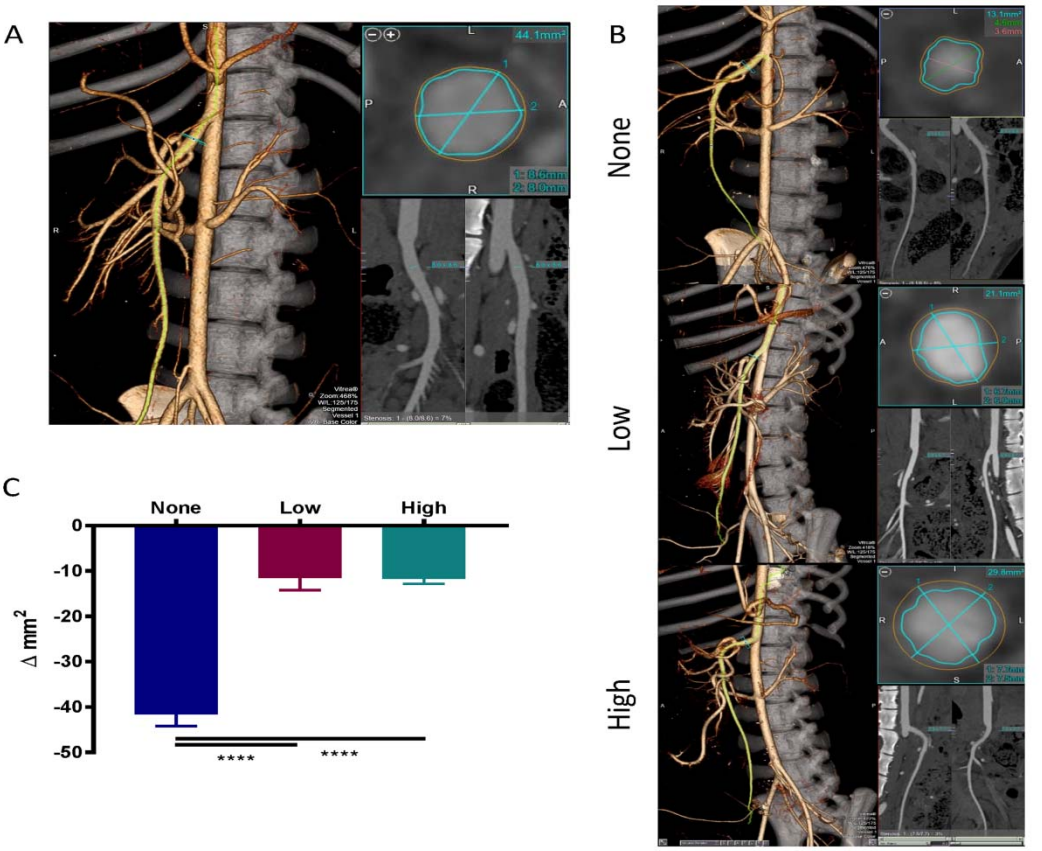


Figure 5. Apoptosis within the intestine. Representative 20x images of TUNEL staining is shown in (A) which reveals a higher amount of apoptotic cells within the epithelium and lamina propria of the jejunum from animals with no IV fluids. Blue indicates all nuclei (DAPI) while green is labelling apoptotic (TUNEL) nuclei. Scale bar is 50µm. (B) Quantitative Western blotting reveals a significantly higher amount of caspase 3 in the intestine of animals that did not receive IV fluid (P=0.0139, n=5, 5, and 6 in none, low, and high, respectively)

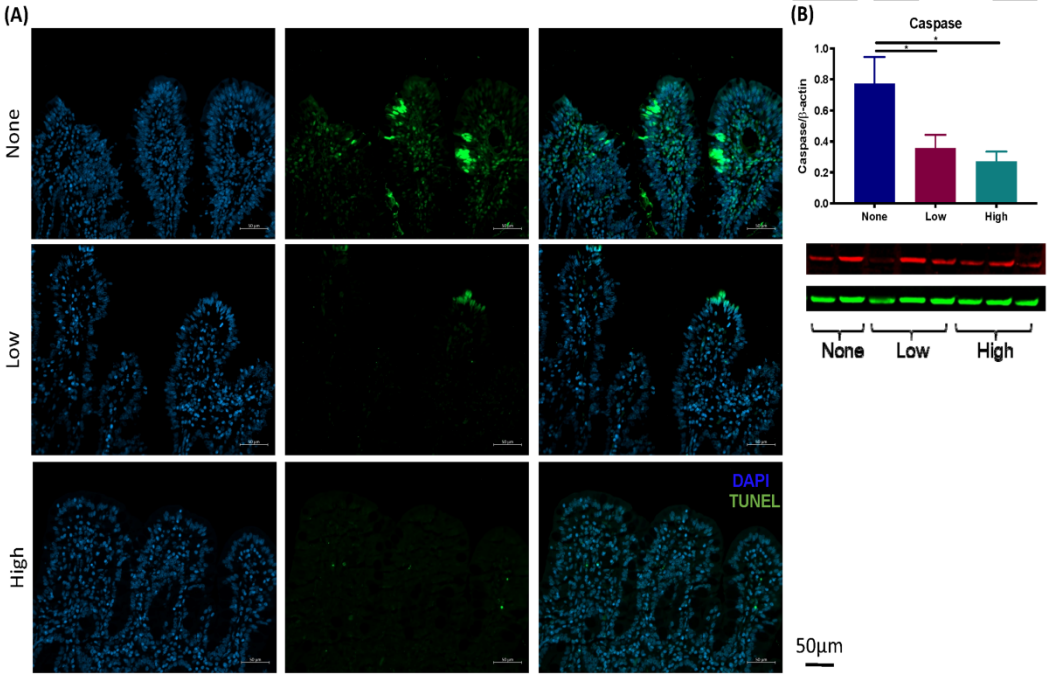
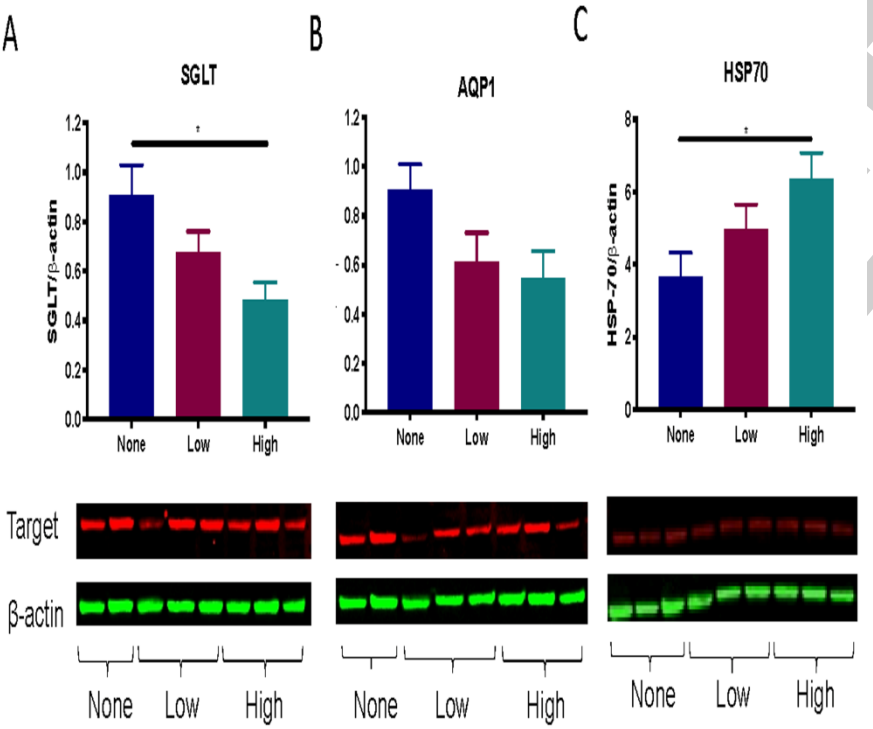


Figure 6. Gut Protein Western Blotting. Significantly lower protein for the active transporter SGLT1 (A) was seen in the high group, with differences in AQP1 (B) not statistically different. Higher HSP70 levels (C) were also seen with large amounts of IV fluids (n=5, 5, and 6 in none, low, and high, respectively).



Operational Advantages of Enteral Resuscitation Following Burn Injury in Resource-Poor Environments

Palatability of Commercially Available Solutions

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ABSTRACT

Background: In recent combat operations, 5% to 15% of casualties sustained thermal injuries, which require resource-intensive therapies. During prolonged field care or when caring for patients in a multidomain battlefield, delayed transport will complicate the challenges that already exist in the burn population. A lack of resources and/or vascular access in the future operating environment may benefit from alternative resuscitation strategies. The objectives of the current report are 1) to briefly review actual and potential advantages/caveats of resuscitation with enteral fluids and 2) to present new data on palatability of oral rehydration solutions. **Methods:** A review of the literature and published guidelines are reported. In addition, enlisted US military active duty Servicemembers (N = 40) were asked to taste/rank five different oral rehydration solutions on several parameters. **Results and Conclusions:** There are several operational advantages of using enteral fluids including ease of administration, no specialized equipment needed, and the use of lightweight sachets that are easily reconstituted/administered. Limited clinical data along with slightly more extensive preclinical studies have prompted published guidelines for austere conditions to indicate consideration of enteral resuscitation for burns. Gatorade® and Drip-Drop® were the overall preferred rehydration solutions based on palatability, with the latter potentially more appropriate for resuscitation. Taken together, enteral resuscitation may confer several advantages over intravenous fluids for burn resuscitation under resource-poor scenarios. Future research needs to identify what solutions and volumes are optimal for use in thermally injured casualties.

KEYWORDS: *burns; prolonged field care; resuscitation; enteral fluids; rehydration solutions*

Introduction

Burn injury in conventional warfare generally accounts for 5% to 15% of military casualties.[1] Thermal injuries lead to hypovolemia, shock, systemic inflammation, and organ dysfunction and, without early intervention, can even lead to death.[2] Compared with civilian patients, combat casualties with burns are characterized by a higher percentage of full-thickness burns and a higher incidence of inhalation injury.[3–6] The extent of total body surface area (TBSA) burned influences the systemic inflammatory reaction as well as burn wound healing which represent important predictors of patient survival. Among the side-effects associated with burns, reduced plasma volume, tissue perfusion, and resultant ischemia can lead to multiorgan dysfunction (MOD), which is often lethal.[7–10]

To treat burn shock–related hypovolemia and MOD, early and aggressive intravenous (IV) fluid resuscitation has drastically improved outcomes during the past several decades.[11–13] Despite recommendations from the American Burn Association,[14] there is significant variability in how patients are resuscitated. However, there is clear consensus that IV resuscitation should be initiated early, which may prove difficult in resource-poor settings.

According to the North Atlantic Treaty Organization (NATO), prolonged field care (PFC) is defined as field medical care applied beyond "doctrinal planning time-lines" by a NATO Special Operations combat medic in order to decrease patient mortality and morbidity.[15] By definition, PFC uses limited resources and is likely to become a more frequent occurrence as a result of future conflicts in multidomain battlespaces and dense urban areas. From a logistical standpoint, both vascular

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access and large volumes of heavy, sterile fluid may not be feasible in these situations. Transport times for burned Warfighters may last several days, which will exacerbate burn-related complications mentioned earlier.[6] The initial 72 hours are particularly critical for burn resuscitation, which begets the need for alternative resuscitation strategies in military relevant PFC scenarios and multidomain battle arenas.

To this end, oral/enteral resuscitation techniques have been proposed in other austere environments,[16,17] including PFC.[18] Despite the promise of leveraging the body's natural mechanism of hydration, there is a large gap in clinical and operational feasibility. The purposes of the current report are (1) to review previous studies examining enteral resuscitation in burn injury and published guidelines on its implementation and operational advantages in resource-poor settings and (2) to evaluate enlisted military opinions on the palatability of currently available oral rehydration solutions.

Methods

Literature Search

A PubMed query to the National Library of Medicine (https://www.ncbi.nlm.nih.gov/pubmed) was performed on 26 June 2018 to include all combinations of the terms "oral," "enteral," "resuscitation," and "burn" as described in the results. Studies were triaged for their relevance, and publications not appropriate for discussion (e.g., author line included 'Burns') were omitted. Review papers were examined thoroughly for their related citations. Emphasis was placed on any guidelines or logistical /operational suggestions for the implementation of enteral fluids in burn injury.

Palatability Study

Forty US Army active duty Servicemembers willingly participated in this study to evaluate the palatability of five oral rehydration solutions. This study was approved by the US Army Institute of Surgical Research, Regulatory Compliance Division after the research was deemed exempt from human use oversight in accordance with 32 Code of Federal Regulations 219.101(b)(6). ID numbers were assigned to each individual for deidentification. Clear instructions were given prior to taking part in the study, which included a station with a pencil, a survey (Figure 1), and randomized five sample cups (4 oz.) that were solid colored with a solid colored lid to prevent the participant from formulating opinions based on the color of the solutions. No-sodium crackers and a cup of water were also placed at each station to cleanse the palate before trying the next sample cup. The comments section shown in Figure 1 proved to be unfruitful.

The five rehydration solutions tested were: the newer formulation of the World Health Organization's oral rehydration solution (WHO), Drip-Drop®, CeraLyte 70®, CeraSport®, and Gatorade®. Gatorade was included as a positive control for palatability, as opposed to suspected optimal efficacy for burn patients. All of these solutions are commercially available with varying ingredients and osmolarity when rehydrated as directed by the manufacturer (Figure 2). The national stock numbers are 6505-01-197-8809, 6505-01-646-2692, 6505-01-420-9275, 6505-01-576-2674, and 8960-01-114-2101 for WHO, Drip-Drop, CeraLyte 70, CeraSport, and Gatorade, respectively. Only lemon-flavored solutions were chosen to reduce bias toward a certain flavor choice (only not possible

FIGURE 1 *The oral rehydration solution survery that was handed out to active duty Servicemembers. A scale of 1 to 5 was used to rate each solution on flavor profile (top) and consumer aspects (middle). Additionally, Servicemembers were asked to rank the solutions from 1 to 5 (bottom).*

Oral Re-Hydration Taste Survey

Instructions: You will have the opportunity to try 5 types of rehydration fluids labeled A-E. Eat a cracker before sipping each rehydration drink and rate the drinks based on cup letter. You are welcome to try each drink more than once and in no particular order. At the end please rank the drinks from least to most favorite and provide additional comments on the drinks.

Please rate the following drinks from 1-5.

1) Non-Palatable 2) Somewhat Tolerable 3) Tolerable 4) Palatable 5) Highly Palatable

	How would you rate the saltiness of this drink?	How would you rate the sugariness of this drink?	How would you rate the viscosity of the drink?	How would you rate the overall taste of this drink?
Cup A				
Cup B				
Cup C				
Cup D				
Cup E				

Please rate the following drinks from 1-5.

1) Disagree 2) Somewhat Disagree 3) Neither agree nor Disagree 4) Agree 5) Strongly Agree

	I enjoyed this drink.	I would drink this regularly.	I would purchase this drink.
Cup A			
Cup B			
Cup C			
Cup D			
Cup E			

Please RANK the 5 rehydration drinks from 1-being least favorite to 5-being most favorite.

	Rank
Cup A	
Cup B	
Cup C	
Cup D	
Cup E	

Please provide additional comments:

FIGURE 2 *The packaging of the different powders for the oral rehydration dirnks are shown at the top. Interestingly, the World Health Organization and the CeraLyte 70 packages are forumlated for 1L reconstitution, while the others are for 0.50 to 0.59L. The corresponding table gives osmolarity and ingredients of the different solutions after they are reconstituted according to the manufacturer's instructions. Prices obtained from Amazon.com product listings and may be found in bulk packaging or at a lower cost elsewhere.*

	WHO ORS	Gatorade	Drip Drop	Cerasport	CeraLyte 70
Osmolarity (mOsm/L)	245	365	235	135	260
Sodium (mEQ/L)	75	23.5	60	35	70
Potassium (mEQ/L)	20	3	20	10	20
Sugars (g/L)	14	58	30	0	0
Magnesium (mg/L)	0	0	13	0	0
Zinc (mg/L)	0	0	5	0	0
Citrate (mEQ/L)	65	-	150	5	10
Calories (kcal/L)	55	200	130	40	160
Weight (g/L)	20.5	60	42.19	42	50
Cost (price/L)	$0.40	$2.28	$5.81	$3.50	$7.13

with WHO). Each rehydration solution was prepared according to manufacturer's instructions and presented to the participants at room temperature. The survey used (Figure 1) included questions on saltiness, sweetness, viscosity, and taste. Additionally, more subjective measures of palatability were also included, as well as a final ranking of each of the five solutions.

Statistical Analyses

GraphPad Prism was used for statistical analysis and graphic representation of data. Analysis was completed with a two-way ANOVA for both the drink characteristics and consumer-based questions. All data are presented as mean ± SEM. Significance was set at $p < .05$.

Results and Discussion

Literature Search Results

The two searches using key words "oral resuscitation burn" and "enteral resuscitation burn" resulted in 162 unique publications, of which 130 were excluded. For example, articles written in another language (n = 34) were excluded, as were articles that appeared because "burn" was in the author affiliation (n = 5) or used "oral" as a method description (n = 3). Many articles were excluded because of irrelevance to the topic, which included publications that focused on timing and volumes of nutrition administration (n = 31), airway management (n = 14), conditions other than burns (e.g., Steven Johnson syndrome, n = 15), or wound healing (n = 3). Other articles not pertinent to the discussion were reviews that did not focus on the topic of burn resuscitation (n = 25). Of the 32 studies selected for inclusion, there was a fairly even distribution of preclinical studies (n = 13), clinical studies (n = 8), and review papers focused on austere environments or underdeveloped countries (n = 11).

Clinical evidence focusing on enteral resuscitation was discussed as early as 1950, as an National Institutes of Health–funded study advocated for the use of oral saline as a standard procedure for the treatment of burn shock.[19] Subsequently, oral fluids were shown to be superior to IV fluids in patients.[20] Some reports have suggested that burns up to 45% TBSA may be treated with oral fluids,[21] and there have been calls to perform a trial of oral fluid resuscitation in larger burns.[22,23] Currently, the limited existing clinical evidence has prevented widespread use of enteral fluids in burn injury, although complications of this strategy have been minimal.[24] A more recent study provided type 1 evidence comparing enteral resuscitation to IV fluids in a randomized, controlled fashion,[25] and found enteral fluids to be equally effective for treating acute kidney injury with increased urine output on day 3 in the enteral fluid group. Still, the authors faced difficulty in the recruitment of subjects for the enteral fluid group, leading to a small sample size.

The idea of supplementing IV fluids with oral fluids was brought up in one study of three burn patients that showed a reduction in the requirement of IV fluids by 58%.[26] This study calculated enteral fluids as part of the total fluid volume needed in these patients, which is not typical. For example, while over half of the burn centers queried in the United Kingdom used oral/enteral fluids, only about one-fifth of respondents thought the approach of oral/enteral fluids to be effective and included them in the volume formula.[27] To the contrary, the joint International Society for Burn Injury and American Burn Association survey revealed that greater than 80% of respondents indicated oral formulas to be working for burns.[28] However, this study also revealed a general lack of enthusiasm for the use of enteral fluids, as there was a significantly lower response rate for questions about oral resuscitation versus the other categories.

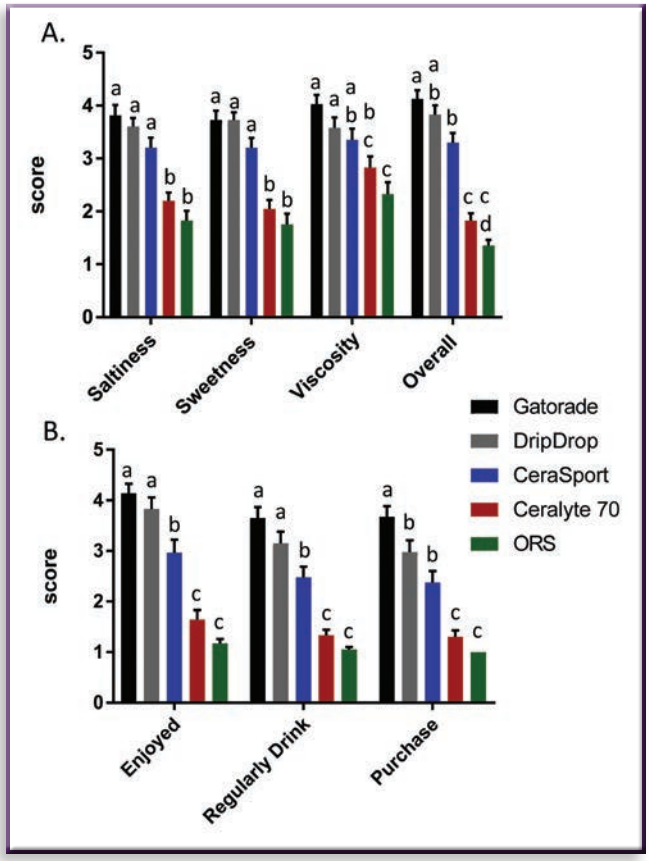
To summarize, preclinical studies of oral resuscitation largely conclude that the strategy is likely safe and efficacious, but protocols need to be optimized and standardized. A seminal animal study demonstrated absorbance of greater than 90% of the infused oral rehydration solution (ORS) in a 40% TBSA burn wound pig model.[29] More recently, a similar model was used to show efficacy in reversing acute kidney injury.[30] Both of these studies used the WHO-ORS, which has been used with great success in underdeveloped nations for the treatment of severe dehydration.[31] The ease of use for this solution resulted in large-scale implementation and has since saved thousands of childrens' lives from afflictions such as cholera. However, there are many other ORS formulations that are widely available and efficacious, many with additives that may prove beneficial such as pyruvate solutions.[32,33] In fact, one study showed superiority of pyruvate oral solutions over a citrate-based solution in terms of intestinal blood flow.[34] There is also a distinct possibility that incorporating pharmacological agents (e.g., opiates or cholinomimetics) may also improve the absorption rate of ORS[35]; however, this needs to be studied in further detail.

Palatability of Resuscitation Fluid

The use of ORS in a conscious patient may have low application if the palatability is undesirable. To that end, results of the taste test performed in the current study are shown in Figure 3. A similar scoring trend was seen for all questions that asked about flavor (Figure 3A) and consumer variables (Figure 3B). Specifically, Gatorade and Drip-Drop were the highest ranked rehydration solution across all variables and were consistently ranked higher than CeraLyte 70 and WHO-ORS ($p < .0001$). Twenty of the 40 active duty Servicemembers ranked Gatorade as their favorite, with 16 members preferring Drip-Drop over the other solutions. No participant ranked these products as tied. In regard to saltiness, sweetness, viscosity, and overall taste, WHO ORS was found to be the lowest ranked, with 72.5% of participants rating the salt content in WHO ORS to be highly unpalatable. Gatorade was selected as the overall preferred solution because 60% of participants stated that as a consumer they would purchase this solution regularly. Drip-Drop was also observed as a solution the participants would purchase regularly, with 37.5% of participants claiming they would buy it regularly. While participants determined that both CeraLyte 70 and WHO ORS were not palatable enough to be purchased regularly, only 22.5% of participants claimed that they would purchase CeraSport regularly as a consumer based on taste.

Palatability is a major consideration when employing enteral fluids, as a rehydration solution will not be effective if not ingested. While previous studies have examined the incidence of vomiting in burn patients to explore what percentage of patients would be candidates for enteral resuscitation,[36] studies that have explored enteral fluids in patients did not consider vomiting to be contraindicated.[25,26] Moreover, increased palatability should not be made at the expense of efficacy.

FIGURE 3 *Results from the taste test. The five oral rehydration solutions are statistically binned in different groups, which are denoted by the letters above each bar (i.e., for saltiness, Gatorade, Drip-Drop, and CeraSport are in one bin and CeraLyte 70 and ORS are in the other bin). Both Gatorade and Drip Drop consitently ranked higher than WHO-ORS and CeraLyte 70 in each category ($p < .0001$) for both flavor and consumer profiles. CeraSport was rated in the middle, and often higher than WHO-ORS and CeraLyte 70 ($p < .001$) for every category except for viscosity (CeraSport versus CeraLyte 70, $p = .2453$).*



Specifically, Gatorade is likely the highest scored in the current study because of its sugar content, which also makes it hyperosmotic (Figure 2). The high sugar and sodium content increases hyperosmolarity and hurts the efficiency and efficacy of this solution, which may only be exacerbated in the insulin-resistant burn patient. For this reason, other solutions that are currently commercially available (e.g., Drip-Drop) may optimize palatability and efficacy.

While osmolarity on extreme ends of the spectrum may be recognizable, it is worth noting that optimal osmolarity for burn injury has not been studied in detail. Indeed, the solutions examined above have had much more extensive study in other conditions (dehydration due to heat, exercise, dysentery, cholera, etc.). The WHO-ORS used in this study is the more recent version that had osmolarity reduced, which is still controversial even for the use in cholera-mediated diarrhea.[37] Different agents such as amino acids and starches that promote absorption in the large intestine have also been recommended in this population.[38,39] Although diarrhea is unlikely to be an issue in the burn patient, inferences of the best solution are largely drawn from experiences in other conditions.

Additionally, palatability would not be an issue in the case where a nasogastric (NG) tube is placed. On the other hand, the volume delivery of fluids via an NG tube may be limited by gastric emptying and reduced intestinal motility. As mentioned earlier, there may be other solutions that prove to be more effective at lower volumes. For example, the CeraLyte 70 solution examined herein uses rice-based carbohydrates as opposed to glucose. In theory, this may facilitate absorption through the sodium-glucose transporter along the length of the small intestine as opposed to the most proximal portion of the duodenum. Additionally, the absorptive capacities of the large intestine may also be leveraged with additives such as amylase-resistant starches.[40] We believe enteral fluids should be viewed as an untapped potential for resuscitation strategies in the resource-limited or austere environment and for reducing IV fluid requirements.

One final consideration on palatability is the environmental exposure to the packets and reconstituted solutions. PFC scenarios that preclude IV fluid delivery will likely also not have refrigeration for ORS fluid. While these sachets of ORS powder are very stable in extreme (i.e., hot/cold) environments, in the present study they were given at room temperature, but in future combat scenarios their administration may only be influenced by their solubility at encountered temperatures. In this regard, previous taste tests of ORS have shown that subjects have a preference for solutions when they are cold versus the same solution given at room temperature (G.C. Kramer, unpublished observations). While, again, this may be circumvented by the use of an NG tube, both of these delivery methods may increase the incidence of vomiting seen in burn patients receiving enteral fluids

Published Guidelines for Low-Resource Scenarios

Despite the lack of evidence comparing different volumes or types of enteral fluids, there are published guidelines for their use in austere environments or in prolonged field care scenarios.[41–46] Also, considering the recent substantial IV fluid shortage due to the destruction of Puerto Rican manufacturing plants by Hurricane Maria, it is prudent to develop enteral strategies for resuscitation. In response to this shortage, oral resuscitation guidelines were recently published as an alternative in cases of mild to moderate dehydration recommending sips every 3 minutes.[47] While this approach is likely insufficient in instances of severe dehydration (as in burns), there is precedent for oral resuscitation. Amazingly, neither the anesthesia handbook for the International Committee of the Red Cross nor the resuscitation first aid guidelines for the International Federation of Red Cross mention the use of enteral fluids in burn injuries. With this in mind, however, the following section concentrates on burn field guidelines published for resource-poor settings.

The recent PFC fluid working group suggested that oral fluids are feasible in patients with 15% to 40% TBSA burns, which may represent a critical burn size in which intervention would have a clinical benefit.[18] It is noted that reports of the use of oral resuscitation have been effective for larger burns.[16,41] Published guidelines in austere environments provide a good overview of potential solutions, from those studied in this report, to chicken broth and apple juice.[44] For volume, the suggestion was to take frequent sips with the goal of ingesting roughly 1 to 2L per hour,[44] with resumption a few minutes after vomiting if it occurs. Interestingly, this study also suggests that overresuscitation as seen with IV resuscitation is unlikely to happen, further promoting the need for a randomized, multicenter study.

Subsequently, specific guidelines on the care of burns in PFC situations have been published.[42] These guidelines explicitly state that IV resuscitation with isotonic fluids is the best option. However, the maximum burn size recommended for enteral resuscitation is less than 30%TBSA, as is the maximum rate of 300 to 500mL/h infusion through an NG tube. Importantly, these recommendations include that plain water is ineffective and may be dangerous, leading to side-effects like hyponatremia. As such, both of the published guidelines mentioned give instructions on how to make a homemade ORS using potable water and sugar, salt, and baking soda or even mixing different proportions of commonly available IV solutions. Similar formulas with different starting points have been given elsewhere, which also mentioned that if a weighing scale is not available, ORS should be prepared to have a similar taste as that of tears.[45] This and other guidelines also mention specific drinks to avoid such as high–sugar content drinks and coffee or other diuretic drinks.

Last, one distinct possibility that has been suggested is the potential for enteral resuscitation through slow infusion through the rectum (proctoclysis),[43] which has previously been used in austere environments for hemorrhagic shock.[48] Indeed, one recent animal study has already tested the absorption capability in the large intestine with colonic fluid.[49] The solution used was normal (0.9%) saline administered through a catheter, which may be safe in unconscious patients and avoids limitations of gastric emptying and reduced intestinal motility. On the other hand, the absorptive capacity of the large intestine is not as high as the small intestine. Lacking any clinical data on this strategy for burn resuscitation, its use cannot be recommended at this time.

Using Enteral Fluids: Other Considerations

While leveraging the body's natural mechanism of hydration seems promising from a clinical efficacy standpoint, there are other logistical considerations that confer advantages to enteral resuscitation. The weight carried by medics has been steadily increasing in the past several conflicts. The small intestine can absorb 15 to 20L of water per day.[50] For a 70kg adult with a 40% TBSA burn injury, clinical practice guidelines (2 to 4mL/kg/%TBSA) recommend infusing 5.6 to 11.2L in the first 24 hours. These numbers equate to anywhere from 12 to 24 lb of fluid alone. In PFC and other delayed transport scenarios, access to these volumes of heavy fluids may not be feasible. The lightweight sachets that present the ORS in powder form will help decrease the weight that combat medics have to carry in their ruck. This will help with maneuverability as well as lower fatigue and strain put on the combat medics out on missions.

The technical expertise needed for using enteral fluids is also much lower than for IV fluids. IV fluids assume vascular access is a viable option, which may not always be the case. For enteral fluids, there is not much concern when it comes to sterility, and requirements are a clean source of potable water. The ORS packets are reconstituted very easily with a clearly defined volume of water. The packets could be carried in large quantities with very little concern of keeping these packets in sterile environments. This allows the combat medics as well as any combat lifesavers attached to the platoon to provide rapid treatment in tactical environment. These oral rehydration packets could be placed in each Servicemember's IFAK (individual first aid kit), which would make access to this treatment in the event of burn injury as accessible as a tourniquet. The use of these oral rehydration packets could be discussed and taught before deploying as a part of basic life saver fundamentals taught throughout the military. Another consideration in these scenarios (as well as mass casualty care) is the cost of resuscitation fluids (Figure 2). In addition, these solutions require no specialized equipment or machinery that would further increase cost. As opposed to pumps, poles, and needles, the only other object needed for enteral fluids would be a cup, bottle, or canteen. In short, oral rehydration packets are a low cost yet very effective way to ensure that the Servicemembers down range can receive timely treatment for burn injuries.

Conclusion

While prospective randomized trials for enteral resuscitation are long overdue, the use of the gut for hydration/resuscitation of the moderately burned patient is feasible in resource-poor settings. Guidelines indicate that burns of up to 40% TBSA may be successfully treated in this manner. Furthermore, literature suggests that this strategy may reduce IV fluid requirements, which should also be examined. The current study of oral rehydration fluid preferences based on palatability, revealed that currently available solutions carry sufficient palatability while maintaining the osmolarity of the WHO-ORS, which is important considering its life-saving track record. Taken together, while there are many operational advantages of this resuscitation strategy, many questions on efficacy, volumes, additives, etc. remain unanswered.

Funding

US Army Medical Research and Materiel Command and Congressionally Directed Medical Research Program award #W81XWH-16-0041 provided funding for this project.

Disclaimer

The views expressed in this article are those of the author(s) and do not reflect the official policy or position of the US Army Medical Department, Department of the Army DoD, or the US Government.

Financial Disclosure

The authors have indicated they have no financial relationships relevant to this article to disclose.

Author Contributions

DB and BG conceived the study concept. JL and BG recruited participants and performed studies. DB and MD obtained funding. DB wrote the first draft, and all authors approved the contents of the final manuscript.

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Burn-Induced Reductions in Mitochondrial Abundance and Efficiency are More Pronounced with Small Volumes of Colloids in Swine

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Running head: Burn Resuscitation on Cardiac Mitochondrial Function

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Abstract

Severe burn injury results in systemic disruption of metabolic regulations and impaired cardiac function. Restoration of hemodynamic homeostasis utilizing intravenous (IV) fluids is critical for acute care of the burn victim. However, the effects of burns and resuscitation on cardiomyocyte mitochondria are currently unknown. The purpose of this study is to determine cardiac mitochondrial function in a swine burn model with subsequent resuscitation using either crystalloids or colloids. Anesthetized Yorkshire swine (n=23) sustained 40% total body surface area (TBSA) burns and received IV crystalloids (n=11) or colloids (n=12) after recovery from anesthesia. Non-burned swine serving as control (n=9). After euthanasia at 48h, heart tissues were harvested, permeabilized, and analyzed by high-resolution respirometry. Citrate synthase (CS) activity was measured, and Western blots were performed to quantify proteins associated with mitochondrial fusion (OPA1), fission (FIS1), and mitophagy (PINK1). There were no differences in State 2 respiration or maximal oxidative phosphorylation. Coupled Complex1 respiration decreased, while uncoupled State 4_O, and complex II increased significantly due to burn injury, particularly in animals receiving colloids ($p<0.05$). CS activity and electron transfer coupling efficiency were significantly lower in burned animals, particularly with colloid treatment ($p<0.05$). Protein analysis revealed increased FIS1, but no differences in mitophagy in cardiac tissue from colloid-treated compared to crystalloid-treated swine. Taken together, severe burns alter mitochondrial respiration in heart tissue, which may be exacerbated by early IV resuscitation with colloids. Early IV burn resuscitation with colloids may require close hemodynamic observation. Mitochondrial stabilizing agents incorporated into resuscitation fluids may help the hemodynamic response to burn injury.

Keywords: mitochondria; resuscitation; heart; burn; swine

Introduction

Burn injuries are among the most common type of injuries seen by emergency departments with an estimated 450,000 cases annually across the United States (3, 47). Large TBSA burns result in systemic inflammation and multiple organ dysfunction that can lead to long-term complications and systemic pathophysiological comorbidities (7, 22-25, 42). For example, human and animal studies have shown altered cardiac function (e.g. increased heart rate/cardiac output and perturbations in ventricular activity) (19, 20, 22, 24). These cardiac dysfunctions lead to longer hospitalization stays and require more surgical interventions(21).

To counteract this (and other) pathophysiological consequences of burn injury, aggressive fluid resuscitation has become a critical component of acute burn care which has greatly improved outcomes (11). Barrow and colleagues found that resuscitation within the first two hours after injury improved outcome over patients who received delayed fluid resuscitation (1). While insufficient fluid resuscitation may lead to multiple organ failure and death, over perfusion may also increase morbidity and mortality via "fluid creep" (11, 40). Moreover, resource-poor environments such as prolonged prehospital times and mass casualty scenarios may limit the amount of fluids available. Although volume-sparing strategies (e.g., colloids) have been explored, crystalloids have been preferred because of their low cost and even distribution (48). Partly because crystalloid resuscitation can exacerbate fluid transfer into the interstitial space, there has been a recent push for the use of colloids. In short, the optimal resuscitative fluid for perfusion of organs and tissue post-burn remains controversial (11, 38).

Mitochondria are ubiquitous organelles that play a critical role in bioenergetics and oxygen utilization to maintain homeostasis and normal organ function. As a result of severe burn injury, increased mitochondrial uncoupling contributes to the hypermetabolic stress response (35). Additionally, burn-induced mitochondrial damage in heart tissues correlated with increased oxidative stress (46). While severe burns alter mitochondrial function in tissues such as skeletal muscle (34-36) and adipose tissue (30, 44), mitochondrial function in heart tissues post-burn has not been well studied. Moreover, the effects of fluid resuscitation on

mitochondrial function in heart tissue and burn-induced metabolic responses is unknown. To this end, the purpose of this study was to examine cardiac mitochondrial respiration in a porcine burn model with a preliminary investigation into the effects of early limited resuscitation with crystalloids or colloids. This information can serve as a first step in identifying the role of mitochondrial respiration in cardiac dysfunction post-burn. We hypothesized that burn induces mitochondrial dysfunction in cardiac tissue, which could be ameliorated with low volumes of IV fluids.

Materials and Methods

Animals

We utilized sexually immature female Yorkshire swine (n=32) weighing 41.7 ± 3.0 kg at approximately 4 months old (Midwest Research Swine, Gibbon, MN). All animals screened upon arrival to our institute were free of parasites and infection, and individually housed with ad libitum access to water during a minimum seven-day acclimatization period. 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.

Thermal injury

The procedures for thermal injury and care have been previously described (7, 15). Briefly, after an overnight fast, animals were anesthetized with an intramuscular (IM) injection of tiletamine-zolazepam (Telazol, 6 mg/kg) then intubated and placed on a ventilator with 1%-3% isoflurane. Hair was shaved on the dorsum, flanks, and legs. Standard vascular cut down procedures were conducted to insert two jugular IV lines (for blood sampling and IV fluid resuscitation), which were tunneled to the back of the neck and secured in place. Animals were given an IM injection (0.1 – 0.24 mg/kg) of Buprenex-HCl Sustained Release (Veterinary Technologies/ZooPharm, Windsor, CO), which has been shown to provide analgesia for 72 hours. Full thickness burns were produced by utilizing custom designed brass blocks (5x5cm, and 9x15cm) heated to 100°C which were applied to the skin for 30 seconds (8). This was repeated until 40% of the TBSA was affected, which was covered with Ioban Antimicrobial Incise Drapes (3M, St. Paul, MN).

Fluid resuscitation

All animals recovered in individual metabolic cages and were resuscitated with a limited volume of IV fluids (15 ml/kg/day) within one hour following the burn injury and at 24 hours post-injury. Fluids were given within a 15 minute period on both days, and the volume was based on feasibility in low-resource (e.g.,

prolonged field care) scenarios (45). Animals were randomly assigned to receive crystalloids (Lactated Ringer's or PlasmaLyte) or colloids (5% albumin or fresh frozen plasma (FFP)). While statistical power did not preclude the detection of differences in PlasmaLyte and LR, or Albumin and FFP, we did not observe differences in these comparisons in our analyses. Thus, these groups were collapsed to crystalloids (n=11) and colloids (n=12); however, where appropriate the data is presented to distinguish individual fluid types. To control for the effects of oral/enteral fluids, all animals received and consumed the same amount of oral rehydration solution. Hearts from nine non-burned swine (including 4 sham-burned) served as control. Animal health and behavior were continuously monitored, and light sedation with Midazolam (0.1 – 0.25 mg/kg) was given when needed. Heart rate and mean arterial pressure were manually recorded at pre-injury (baseline), 24- and 48-hours post-injury.

Mitochondria analysis

At 48 hours post-injury, animals were euthanized and tissues from the apex of the left ventricle of the heart were harvested for biochemical and mitochondrial analyses. Within one minute of tissue harvest, approximately 200 mg of fresh heart tissue was placed in an ice-cold mitochondrial preservation buffer (BIOPS; 10mM Ca-EGTA, 0.1 μM free Ca^{2+}, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl2, 5.77 mM ATP, and 15 mM creatine phosphate, pH 7.1). Cardiac muscle tissues were then separated manually by sharp forceps into several small fibers then permeabilized in BIOPS with 5 μM saponin for 30 minutes at 4°C. After permeabilization, approximately 2 mg of cardiac muscle tissue was blotted dry, weighed, and transferred to the chambers of an O2K respirometer (Oroboros Instruments, Innsbruck, Austria) suspended in 2 ml of mitochondrial respiration buffer (Mir05; containing 0.5 mM EGTA, 3 mM MgCl2, 60 mM lactobionate, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 10 mM sucrose, and 1 mg/ml bovine serum albumin; pH 7.1).

A SUIT (substrates-uncouplers-inhibitors-titration) protocol (representative tracings are shown in Supplemental Figure 1- https://doi.org/10.6084/m9.figshare.8251427.v1) was followed to determine each respiratory state using an O2K respirometer (Oroboros Instruments, Innsbruck, Austria) (14, 31). First, the

Leak respiratory state was recorded with the cardiac myofibers alone. Afterward, saturating levels of substrates (octanoyl-L-carnitine (1.5 mM), pyruvate (5mM), malate (2mM), glutamate (10 mM)) were added to determine State 2 uncoupled respiration supported by complex I. Then, ADP (5mM) was added to reach respiration coupled with ATP production that is supported by complex I only (Complex 1). Then 10 mM succinate was added to provide electrons via succinate dehydrogenase, thereby activating the maximal oxidative phosphorylation capacity (Oxphos) supported by complexes I and II of the electron transport chain. Next, 5 μM oligomycin, an inhibitor of the F_O unit of the ATP synthase, was added to induce maximal uncoupled respiration (State 4_O) that is supported by all the complexes of the electron transport chain without ATP production {Chance, 1955 #147}. FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone), a protonophore and an uncoupler of oxidative phosphorylation, was titrated (0.5 μM steps) to determine the maximum oxygen flux (MOF) capacity. Rotenone (0.5 μM) was then added to inhibit complex I of the electron transport chain to induce uncoupled respiration supported by complex II. From these data, the intrinsic mitochondrial function can be calculated by the coupling control flux (CCF:1- (State 4_O/Oxphos)) as a measure of mitochondrial ATP producing efficiency, or electron transfer coupling efficiency. CCF is expressed as a percentage to represent the efficiency of ATP produced for a given amount of oxygen consumed, or successful coupling of the elctron transport chain. For example, a CCF of 70% indicates that 70% of the oxygen utilization was coupled with ATP synthesis {Doerrier, 2018 #146}. In this way, the CCF decreases with uncoupling.. The excess capacity factor (ECF) was calculated by MOF-(State4_O/MOF): this represents the supraphysiological portion of the electron transport chain that may contribute to phosphorylation. Similarly, the Oxphos coupling efficiency was also calculated as Oxphos-(State4_O/Oxphos).

Citrate Synthase Assay

CS activity (a proxy of mitochondrial abundance) (13, 26, 33) was performed on a subset of heart tissues kept for later analysis according to the manufacturer's instructions (Sigma Aldrich, MAK193, St. Louis, MO). Briefly, frozen heart tissues were homogenized in CS Assay Buffer at a 1 mg: 10 μl ratio using a

glass dounce homogenizer. Samples were then centrifuged (14,000 rpm, 15m, 4°C) and the supernatant was transferred to a fresh tube for analysis. Standards and samples were mixed with CS Developer and CS Substrate Mix in a 96-well plate, incubated, and absorbance at 412 nm was measured on a kinetic plate reader, and activity was calculated by subtracting the values at 5 minutes from 20 minutes.

Histopathology

Upon euthanasia at 48h, heart samples were immediately preserved in 10% neutral buffered formalin, embedded in paraffin wax, and sectioned into 4-μm slices. Routine H&E staining was scored on a scale from 0-5 by a blinded histopathologist for severity and distribution of myocardial degeneration hallmarks (e.g., vacuolar degeneration, hyaline casting, myocyte rowing, Anitschkow cells) and edema. Additionally, immunohistochemistry (IHC) for Pink 1 (Abcam, ab23707, Cambridge, MA) was performed by heat-mediated antigen retrieval with 0.01M citrate buffer at 95-98°C for 15m, followed by an endogenous peroxidase block with 0.3%H2O2 for 20 minutes at room temperature. Non-specific IgG was also blocked with 10% horse serum in HBSS for 30m at room temperature, and sections were then incubated with anti-Pink1 (Abcam, ab23707, rabbit polyclonal, at 4μg/ml) and anti p-Parkin (Biorbyt, orb312554, rabbit polyclonal, 1:400 dilution) for 60 minutes at room temperature. Slides were then washed with HBSS and treated with biotinylated anti-mouse secondary antibody (Vector Labs, BA-2000) for 60 minutes. Finally, immunostaining was completed with 30-minute incubation with Vectastain-RTU Kit solution (Vector Labs, PK-7100) followed by 5- 10-minute incubation with ImmPACT DAB (Diaminobenzidine, Vector Labs, Inc., Burlingame, CA) at room temperature. Slides were then counterstained with hematoxylin and dehydrated for cover slipping.

Western blot

Heart tissues from the apex of the left ventricle were homogenized in lysis buffer containing mammalian protein extraction reagent (Thermo Fisher, 78501, Grand Island, NY) and protease/phosphatase inhibitor (Sigma Aldrich, PPC1010, St. Louis, MO). Protein concentration was determined by BCA assay (Thermo Fisher, 23225, Grand Island, NY), and 15μg of protein were separated by SDS-PAGE. Proteins

were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA), which was incubated overnight with primary antibodies at 4°C in 5% non-fat milk+TBST, washed, and probed with an appropriate fluorescent for imaging in an Odyssey™ scanner (Li-Cor, Lincoln, NE). OPA1(1:500 dilution, Novus Biologicals, NB110-55290, Littleton, CO), Mitochondrial fission 1 protein (FIS1, 1:1,000 dilution, Proteintech, 10956-1-ap, Rosemont, IL), Dynamin-related protein 1 (DRP1, 1:2000 dilution, Cell Signaling Technologies, D6C7, Danvers, MA), and mitophagy (Phosphorylated-Parkin, 1:500 dilution, Biorbyt, orb312554, San Francisco, CA, and PTEN-induced kinase 1 (PINK1), 1:500 dilution, Abcam, ab23707, Cambridge, MA) were determined. GAPDH (1:10,000 dilution, Abcam, ab9485, Cambridge, MA) was used to normalize total protein.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism software v7 (San Diego, CA). D'Agostino-Pearson omnibus test for normality was done for all parameters. An ordinary one-way ANOVA with Tukey's or Dunn's multiple comparisons test (as appropriate for normality) with a single pooled variance was performed, with the exception of Western data which compared crystalloids and colloids via Mann-Whitney tests. Correlations were determined using Pearson's r test and a linear regression was used to determine the linear equation. Values are presented as mean ± SE, and samples sizes given in the results and figure legends represent biological replicates. Statistical significance was determined when $p < 0.05$.

Results

Physiological measures

Heart rate (HR) and mean arterial pressure (MAP) were measured at 24h and 48h and presented as a percentage of BL values **(Table 1)**. Although there was a trend for increased heart rate and decreased MAP in animals receiving crystalloids, no significant differences were found among groups or within groups over time.

Heart mitochondrial respiration

Citrate synthase (CS) activity (as a proxy for mitochondrial abundance) was significantly lower in burned animals than control (41.8 ± 3.34 vs 61.4 ± 6.08 nmol/ml/sec; $p<0.01$) (**Fig 1A**). When examining the effect of fluids, the colloid-treated animals showed significantly less CS activity than control (36.3 ± 3.37 vs 61.04 ± 6.08 nmol/ml/sec; $p<0.01$). CS activity in crystalloid-treated animals was not significantly different from control or colloid-treated animals (**Fig 1B**).

The effect of burn on mitochondrial respiratory states was examined irrespective of fluid resuscitation. No significant differences in oxygen flux per mg of permeabilized heart myofibers were found in Leak (4.10 ± 2.6 pmol vs 3.99 ± 2.9 pmol O_2/mg/sec O_2/mg/sec), Oxphos (61.4 ± 3.6 pmol vs 68.1 ± 7.1 pmol O_2/mg/sec O_2/mg/sec) and MOF capacity (66.6 ± 6.3 pmol vs 66.5 ± 4.5 pmol O_2/mg/sec O_2/mg/sec) (data not shown) between burn and control hearts. As shown in **Figure 2A**, Complex 1 coupled respiration supported by complex I was significantly lower in burned animals than control (36.9 ± 3.64 vs 51.4 ± 4.99 pmol O_2/mg/sec; $p<0.01$). Alternatively, state 4_O oligomycin-induced uncoupled respiration was significantly higher in burned animals than control (25.8 ± 2.57 vs 16.6 ± 1.65 pmol O_2/mg/sec; $p<0.05$), as was complex II uncoupled respiration (29.7 ± 1.76 vs 23.3 ± 1.24 pmol O_2/mg/sec; $p<0.05$) (**Fig 2A**).

To determine whether these differences were affected by mitochondrial abundance, the respiratory states were normalized to citrate synthase (CS). Similarly, no differences were found for Oxphos and MOF

capacity (data not shown). However, **Figure 2B** illustrates that the difference in Complex 1 respiration was no longer found when normalized to CS activity. Alternatively, both State 4_O uncoupled respiration (0.765 ± 0.116 vs 0.254 ± 0.058 pmol O_2/mg/sec/CS; $p<0.01$) and complex II uncoupled respiration (0.848 ± 0.088 vs 0.417 ± 0.082 pmol O_2/mg/sec/CS; $p<0.01$) remained significantly higher in burned animals vs control.

Animals were then separated into either crystalloid- or colloid- treated groups to determine the effects of fluids on heart mitochondrial respiration. Again, no significant differences were found amongst groups in Oxphos and MOF capacity (data not shown). As shown in **Figure 3A**, Complex 1 coupled respiration was significantly lower in colloid treated swine than control (32.7 ± 4.69 vs 51.4 ± 4.99 pmol O_2/mg/sec; $p<0.05$), while State 4_O uncoupled respiration (28.1 ± 2.57 vs 16.6 ± 1.65 pmol O_2/mg/sec; $p<0.05$) and complex II uncoupled respiration (32.2 ± 2.69 vs 23.3 ± 1.24 pmol O_2/mg/sec; $p<0.05$) were significantly higher in colloid treated swine than control. When normalized to CS activity (**Fig 3B**), Complex 1 respiration was no longer different amongst groups ($p>0.05$). However, colloid treated animals still showed significantly higher respiration vs control in State 4_O (0.851 ± 0.103 vs 0.254 ± 0.0577 pmol O_2/mg/sec/CS; $p<0.01$, respectively) and complex II (0.924 ± 0.0745 vs 0.417 ± 0.0822 pmol O_2/mg/sec/CS; $p<0.01$, respectively).

Several ratios representing intrinsic functions of mitochondria in terms of mitochondrial efficiency were calculated. Coupling control flux (CCF) calculation revealed that burn-injured animals showed lower ATP producing efficiency than control animals (56 ± 3% vs 71 ± 5%; $p<0.05$, respectively) which was driven by colloid-treated animals (52 ± 4% vs 71 ± 5%; $p<0.05$) (**Fig 4A**). No differences were found in CCF between crystalloids vs control or crystalloids vs colloids. Along those same lines, both the Excess Capacity Factor (55 ± 4% vs. 75 ± 3%) and the Oxphos Coupling Efficiency (52 ±4% vs 73 ± 4%) were significantly lower in colloid-treated animals compared to control. Moreover, a weak but significant positive correlation was found between CS activity CCF ($y=0.004x + 0.431$, $p<0.05$) (**Fig 4B**), whereas a significant negative correlation was found between CS activity and State 4_O uncoupled respiration ($y= -0.254x + 35.68$, $p<0.05$).

Mitochondrial dynamics and mitophagy

H&E images representing mild cardiac degeneration are shown in Figure 5A. While multifocally, there were instances of myocyte nuclear rowing, necrotic cardiomyocytes and inflammation, there were no significant differences in histopathologist scoring between the groups. While western blot analyses revealed no differences in mitochondrial fusion (OPA1) proteins, FIS1proteins were higher (**Fig 5B**) in colloid-treated vs crystalloid-treated animals (6.44 ± 0.404 vs 5.23 ± 0.466 r.l.u. (relative light units); $p<0.05$). However, the ratio of phosphorylated DRP1 to total was not different amongst the groups (Figure 5C) with ratios of 0.51±0.03, 0.52±0.03, and 0.48±0.03, in the control, crystalloid, and colloid groups, respectively.

Mitophagy post-burn was examined via Western blot and immunohistochemistry (**Fig 6)**. Protein levels of PINK 1 (**Fig 6A**) were significantly lower in colloid-treated animals compared to crystalloid-treated animals (0.021 ± 0.001 vs 0.025 ± 0.003 r.l.u.; $p<0.05$). PINK1 immunostaining (**Fig 6B)** also reveals a lower amount of punctate staining within the hearts of animals treated with colloids post-burn. However, analysis of p-Parkin revealed no difference amongst the groups (data not shown).

Discussion

Severe burn injury results in a systemic hyperinflammatory response leading to prolonged metabolic abnormalities (22, 24, 37) and altered cardiac function (19). While mitochondrial damage has been observed following severe burns (19, 46), it is unclear whether mitochondrial respiration in the heart is altered post-burn as it is in other organs. Herein, we sought to determine mitochondrial respiration by high-resolution respirometry following severe burn injury and identify potential effects of limited-volume crystalloid and colloid fluid resuscitation. Cardiac muscle from burned animals displayed reduced mitochondrial activity and increased uncoupling within 48 hours after severe burn injury, which was especially prominent after resuscitation with colloids. To our knowledge, this is the first study to examine mitochondrial function in response to different types of fluid resuscitation following severe burn injury.

It is well known that the metabolic stress response to severe burn trauma occurs in a time-dependent manner (24, 50). Severe burn survivors experience substantially increased heart rate and oxygen demand beyond one year after injury(28, 29). The lack of burn-induced change in HR and MAP in our study may be explained by the timing of the study, and 48 hours is within the critical point where the stress response to severe burn injury is transitioning from the "ebb phase" to the "flow phase." On the other hand, myocardial depression occurs within a couple of hours after burn injury and resolves in 2-3 days (20). While we did not harvest tissues in the first several hours post-burn, the lack of change in blood pressure at 24 hours does not preclude alterations in cardiac output if peripheral resistance compensates. However, it has been shown that (despite normal ejection fractions) crystalloids alone transiently lower cardiac index in the 12-24 hour time frame when compared to colloids (16). In this regard, we cannot rule out that reduced mitochondrial number or efficiency is occurring in the current study in the lack of physiologically significant impairments in cardiac function.

Similarly, the timing of infusion for each fluid type is also a critical component of burn resuscitation. Colloids are rarely used in the United States within the first 12 hours of burn injury, as there remains great disagreement whether colloids are effective (2, 11, 18) in burn resuscitation. While colloids have been touted

as great intravascular volume expanders by increasing the colloid osmotic pressure, concerns over osmotic pulls into the interstitial space have prevented leveraging their short-lived effects (39). This is of special importance given the capillary leak that occurs in the early post-burn period, even in non-burned tissues. While the recommended use of colloids by the American Burn Association is 12 – 24 hours after burn injury (or as a rescue technique) (32), here we investigated colloid infusion alone one-hour after burn injury. The effects of colloids on hemodynamic responses have been reported as short-lived compared to longer effects on pulmonary edema (16). As such, while we cannot rule out an effect of intravascular volume changes affecting cardiomyocyte respirometry in the current study, we would imagine this effect to be short-lived. The volume-expanding capabilities of colloids are especially attractive in resource-poor environments (e.g., mass casualties, prolonged field care in military scenarios) and may also become crucial if natural disaster-related IV crystalloid shortages become a reality again, as happened due to the destruction of Puerto Rican manufacturing facilities by Hurricane Maria.

Burn-induced mitochondrial damage has been indicated by circulating mitochondria DNA fragments(46), dysregulation of calcium(27), and loss of mitochondrial membrane integrity(52). In this study, we found burn-induced mitochondrial damage evidenced by altered activity of the electron transport chain through high-resolution respirometry. The decrease in complex I function (**Fig 2A**) may contribute to unmet energy demands in heart tissues following burn injury which, if prolonged, could cause organ dysfunction. The mechanism responsible for altered complex I function in burn injury has not been established; however, studies in sepsis suggested that the deficiency in complex I activity could be a result of a deficiency of nitric oxide synthase(5) and decreased tyrosine phosphorylation(53). Although complex I function was inhibited, the maximum oxidative phosphorylation (Oxphos) capacity was unchanged with burn injury, which can be attributed to the increased activity of complex II (**Fig 2A**). Complex II upregulation may be a rescue compensatory response in order to meet energy deficits associated with decreased complex I activity. While we observed this within 48h of burn injury, it is unknown how long complex II would sustain this increased activity, as chronic hypermetabolism continues post-injury.

We also found increased maximal uncoupled respiration (State 4_O) after burn injury, indicating that oxygen utilization was shifted from ATP production to other mechanisms which do not produce ATP (**Fig 2A**). This increase in State4o respiration has been postulated to demonstrate damaged mitochondria (4), and has been seen in both skeletal muscle and fat tissues following severe burns (34, 35, 44). This may be another mechanism that contributes to an insufficient meeting of the energy demands of the high metabolic activity of heart tissues. This resulted in a lower electron transfer coupling efficiency in burninjured animals (particularly with colloid-treatment (**Fig 4)**) which is indicated by the several different ratios that represent intrinsic function and are independent of the number of mitochondria in tissues.

In this regard, not only does severe burn injury result in fewer mitochondria, but the remaining mitochondria are less efficient at producing ATP. Moreover, the phenomenon of decreased mitochondrial number and electron transfer coupling efficiency appear to go hand-in-hand, as citrate synthase activity correlated with State 4_O uncoupled respiration and CCF (**Fig 4**). The correlations suggest that heart tissues that had more mitochondria were less "leaky" and were also more efficient at producing ATP. Thus, the preservation of healthy mitochondria appears to be protective of mitochondrial function and may be an attractive target at improving bioenergetic demands of the heart following traumatic injury. This shift from coupled (with ATP production) to uncoupled respiration was suggested to be a protective mechanism as stated by Brand and Esteves who found strong evidence that "mild" uncoupled respiration attenuates mitochondrial ROS production and protects against cellular damage(6). However, the determination of "mild" versus "high" uncoupled respiration hasn't been clearly defined, and the functional meaning of elevated uncoupled respiration in heart tissues following severe thermal injury is not clearly understood.

To investigate potential mechanisms responsible for changes in mitochondrial abundance, we analyzed OPA1 and FIS1 proteins and found that heart tissues of colloid-treated animals contained significantly higher FIS1 protein than crystalloid-treated animals. However, as an indirect measure of fission we aimed to analyze the activated state of DRP1, and found no differences (Figure 5C). We believe this could be due to a temporal effect (i.e., the 48hour timepoint employed was after most active changes in mitochondrial fission). Greater fission in colloid-treated animals may result in significant changes in

mitochondrial morphology *in vivo* compared to non-burned controls and may at least partly explain the decrease in CS activity. Counterintuitively, this decrease could not be explained by mitophagy, as we found no differences in phosphorylated parkin, however this could again be due to the temporal nature of mitophagy, as the fact that colloid-treated animals showed less PINK1 levels (**Fig 6**) could indicate mitochondrial damage, and an accelerated mitophagy had occurred just prior to 48 hours,. Previously, PINK1 and parkin increases have been described as beneficial by clearing damaged organelle and reducing ROS production (17, 43) and maintaining mitochondria quality control(12). Lower PINK1 may also suggest poorer quality control and cardioprotection with colloid treatment following severe burns, but whether this holds true in our model is unclear.

The mechanistic basis for increased uncoupling remains a worthwhile area of study. It has been shown that damage-associated molecular patterns derived from mitochondria help mediate cardiac dysfunction post-burn, which can be blocked with estradiol (51). While we used female swine (for simply logistical reasons) they were pre-pubertal, and estrogen may not explain the mild response of the heart to 40% TBSA. However, this does not mean that findings are necessarily applicable to males. Moreover, the previous study also implicated cardiac apoptosis and inflammation, which has also shown to be time dependent (9, 10). As one of the cytokines implicated in the acute inflammatory response to burn, IL-6 has also been shown to induce mitochondrial fragmentation of myoblasts *in vitro* (41)and may contribute to mitochondrial dysfunction in this model. Additionally, while hyperglycemia has been shown to induce cardiac mitochondrial damage in other burn models (49), we did not see any difference in circulating glucose levels in the two groups (data not shown). Future mechanistic studies of mitochondrial dysfunction in the heart will incorporate a model that can be directly tied to changes in cardiac output or ejection fraction.

There are some limitations to our study worth mentioning. First, as this study was designed to examine other organ systems, we were not able to measure heart functions (e.g., ejection fraction, stroke volume, cardiac output) to correlate the effect of mitochondrial health and heart function *in vivo*. Secondly, due to technical limitations, we were not able to directly measure real-time ATP production in permeabilized heart tissues. However, we utilized inhibitors to determine respiration that was uncoupled or coupled with

ATP production. Additionally, female swine were used for ease of procurement, and findings may not be attributable to both sexes. Finally, we were only able to study one time-point post-injury. The metabolic dysregulation following severe burns persists long after the initial injury and having both earlier and later time points would provide some valuable insight into the effects of time post-burn.

Despite these limitations, our study showed metabolic dysregulation in mitochondria of the heart illustrating the systemic effect of severe burns. We show that severe thermal injury results in uncoupling of oxidative phosphorylation and a reduction in the abundance of mitochondria in heart tissue, compromising the required energy demand, which is elevated post-burn. Moreover, with a recent interest in using both albumin and plasma for burn resuscitation, it is pertinent to note that cardiac mitochondrial changes were most prominent after colloid infusion. We were unable to determine the specific component(s) in colloid fluids responsible for this undesired effect on mitochondrial function. Our data show that until we know the chronic effect of colloids resuscitation on burn injury, early resuscitation with colloids may necessitate close supervision of hemodynamic status. Additional studies are needed to determine the impact of restoring mitochondrial function and health in preventing multiple organ dysfunction, but our study suggests that mitochondria-targeted therapy may have impactful implications in restoring bioenergetics to improve the recovery process in critical injury.

Acknowledgments

Authors want to thank USAISR Veterinary Support Branch and Laboratory Support for their assistance. T.C. and B.G. was supported in part by an appointment to the Postgraduate Research Participation Program at the USAISR administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and USAMRMC.

Grants

This study was funded by CDMRP award #W81XWH-16-2-0041.

Disclaimers

The opinions or assertions contained here are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army, the Department of Defense, or the United States Government.

Government.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author Contributions

TC– Manuscript preparation, data analysis and interpretation, BG – Research design conceptualization, animal protocol execution, TH – Sample analysis, MD– Animal protocol review, manuscript review, DB– Research design conceptualization, data interpretation, manuscript review.

Abbreviations:

IV-Intravenous

TBSA-Total Body Surface Area

CS-Citrate Synthase

FIS1-Mitochondrial fission 1 protein

DRP- Dynamin-related protein

PINK1- PTEN-induced kinase 1

IM- Intramuscular

FFP-Fresh Frozen Plasma

BIOPS-Mitochondrial Preservation Buffer

Mir05-Mitochondrial Respiration Buffer

SUIT-Substrates-uncouplers-inhibitors-titration

Oxphos- Maximal Oxidative Phosphorylation Capacity

State 4o-Maximal Uncoupled Respiration

FCCP-Carbonyl Cyanide 4-(trifluoromethoxy) Phenylhydrazone

MOF-Maximum Oxygen Flux

CCF-Coupling Control Factor

ECF-Excess Capacity Factor

IHC-Immunohistochemistry

HR-Heart Rate

MAP-Mean Arterial Pressure

Figure Legends

Figure 1. Mitochondrial abundance in heart tissues measured by citrate synthase. (A) A t-test revealed that non-burned control (n=8 female swine) hearts displayed higher CS activity than vs burned animals (n=21 female swine, P=0.011). (B) One way ANOVA revealed that lower citrate synthase activity in cardiomyocytes post-burn is largely attributed to animals that received colloid resuscitation (crystalloids: n=10 female swine, colloids: n=11 female swine, P=0.0059). Squares represent animals that received crystalloids, while triangles represent animals that received colloids. Closed squares represent animals that received Lactated Ringer's, while open squares represent those receiving PlasmaLyte. Closed triangles represent animals receiving FFP, while open triangles represent those receiving albumin.

Figure 2. Mitochondrial respiratory states in non-burned control vs burn animals. (A) T-tests reveal lower Complex 1 respiration (P=0.0016), but higher State 4o (P=0.0012) and uncoupled Complex II (P=0.0023) respiration in burn animals (n=23 female swine) compared to controls (n=8 female swine). (B) After normalization to CS activity, Complex 1 respiration was no longer reduced (P=0.801) while uncoupled state 4o (P=0.0060) and Complex II (P=0.0084) states both remain elevated in burn animals (n=21 female swine) compared to controls (n-5 female swine). Squares represent animals that received crystalloids, while triangles represent animals that received colloids.

Figure 3. Mitochondrial respiratory states in non-burned control after separation of fluid type. (A) One way ANOVA post-hoc testing revealed lower Complex 1 respiration (P=0.009) but higher State 4o (P=0.011) and uncoupled Complex II (P=0.034) in animals that received colloids (n=12) but not crystalloids (n=11 female swine) compared to controls (n=8 female swine). (B) After normalization CS activity, One way ANOVA revealed that Complex 1 respiration was no longer decreased (P=0.8662), but State 4o (P=0.0092) and Complex II (P=0.0067) were still lower after colloids (n=11 female swine) but not crystalloids (n=10 female swine) compared to controls (n=5 female swine). Closed squares represent animals that received

Lactated Ringer's, while open squares represent those receiving PlasmaLyte. Closed triangles represent animals receiving FFP, while open triangles represent those receiving albumin.

Figure 4. Ratios representing intrinsic mitochondrial efficiency. (A) One way ANOVAs revealed that the coupling control flux (P=0.026), excess capacity factor (P=0.025), and oxphos coupling efficiency (P=0.020) were all reduced in burned animals that received colloids (n=12 female swine) but not crystalloids (n=11 female swine) compared to controls (n=8). (B). Linear regression revealed a direct correlation in the amount of mitochondria in heart tissue and their electron transfer coupling efficiency and an inverse relationship showing that more mitochondria in heart tissue correlates with less uncoupled (non-ATP generating) respiration. Closed squares represent animals that received Lactated Ringer's, while open squares represent those receiving PlasmaLyte. Closed triangles represent animals receiving FFP, while open triangles represent those receiving albumin.

Figure 5. (A) H and E staining from all animals post-burn revealed instances of multifocal necrosis (black arrow), with nuclear pyknosis and cytoplasmic hyalinization and loss of cross striations, nuclear rowing of cardiac myocytes (black arrow), Anitschkow cells (black arrow), and mild lymphoplasmacytic inflammation. (B) Representative Western blots for mitochondrial fusion (OPA1) and fission (FIS1) were used for densitometry. Mann-Whitney tests revealed no differences in OPA1 (P=0.219) while FIS1 (P=0.031) was significantly greater in animals treated with colloids compared to those treated with crystalloids (control: n=6 female swine, crystalloids: n=6 female swine, colloids: n=10 female swine. *$p<0.05$.). (C) Alternatively, the phosphorylated portion of DRP1 was not different amongst the groups (p=0.88). Closed squares represent animals that received Lactated Ringer's, while open squares represent those receiving PlasmaLyte. Closed triangles represent animals receiving FFP, while open triangles represent those receiving albumin.

Figure 6. Western and immunostaining for mitophagy. (A) Representative Western blots for PINK1 were used for densitometry. Mann-Whitney tests revealed lower PINK1 levels (p=0.042) in hearts from animals treated with colloids (n=10 female swine) compared to those treated with crystalloids (control: n=6 female swine, crystalloids: n=6 female swine, colloids: n=10 female swine). (B) Representative immunostaining for PINK1 confirms higher amount of punctate staining in sections from crystalloid-treated animals compared to colloid-treated. Closed squares represent animals that received Lactated Ringer's, while open squares represent those receiving PlasmaLyte. Closed triangles represent animals receiving FFP, while open triangles represent those receiving albumin.

Supplemental Figure 1: Representative SUIT protocol from an animal treated with crystalloids (top) and colloids (bottom). Substrates are shown above arrows, while different states are bolded.

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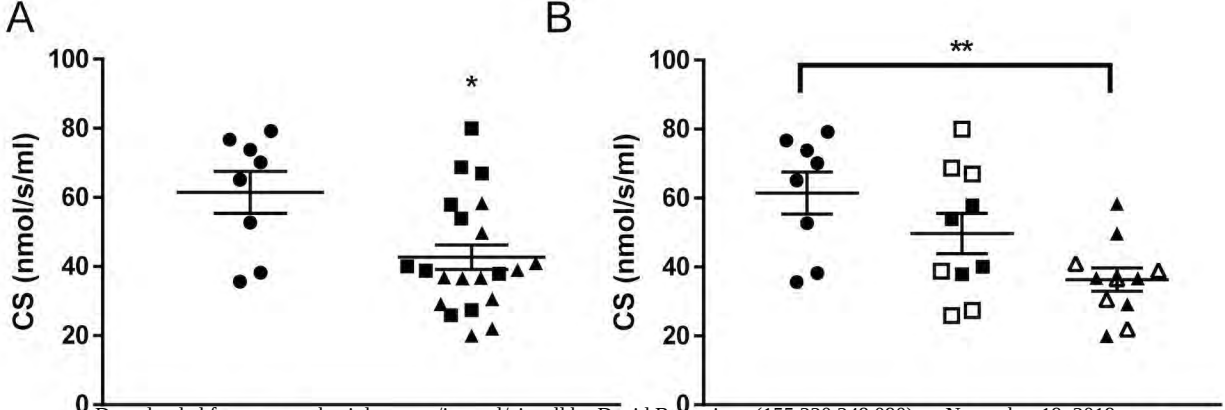
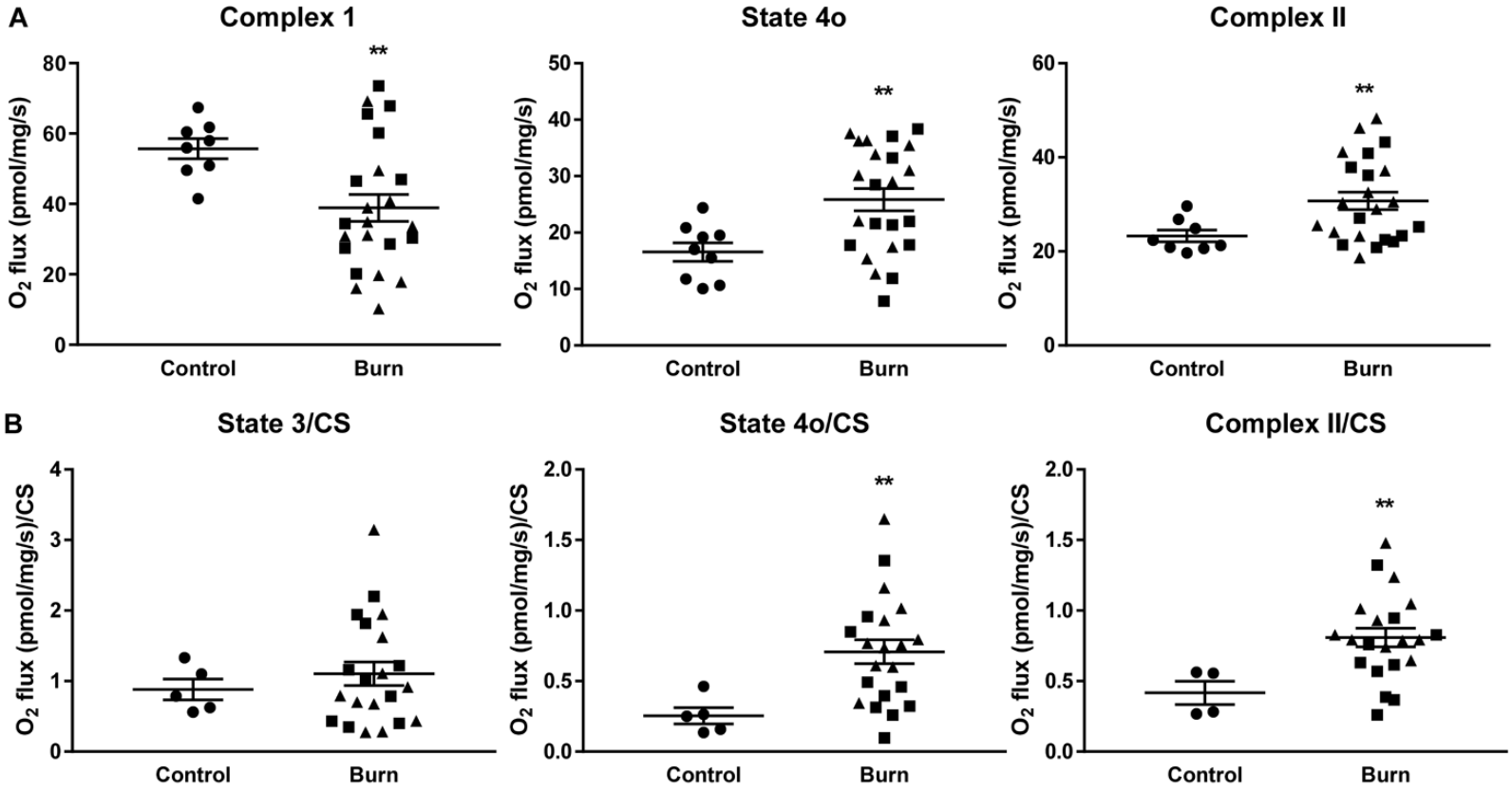
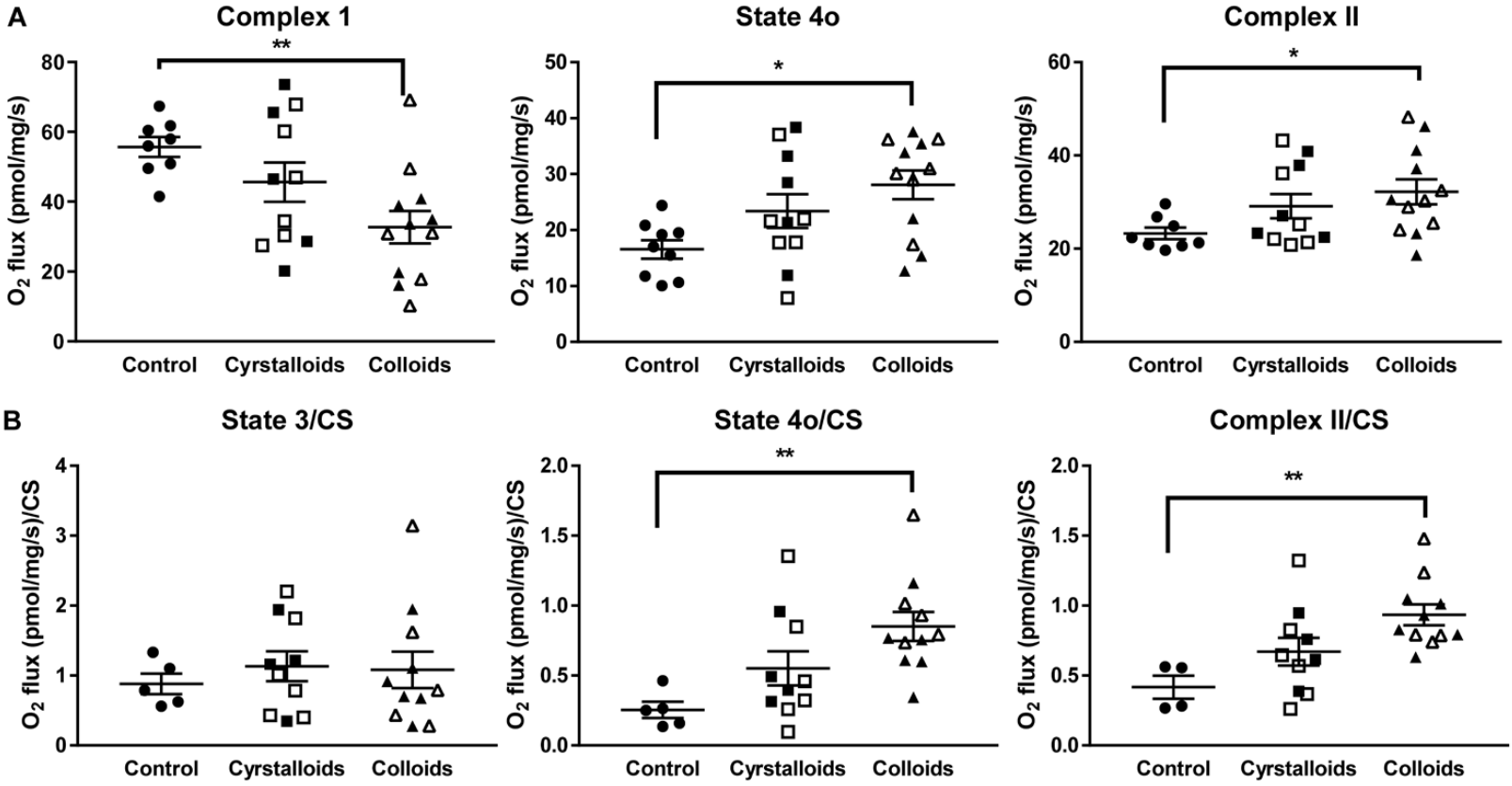
Table 1. Physiological measures

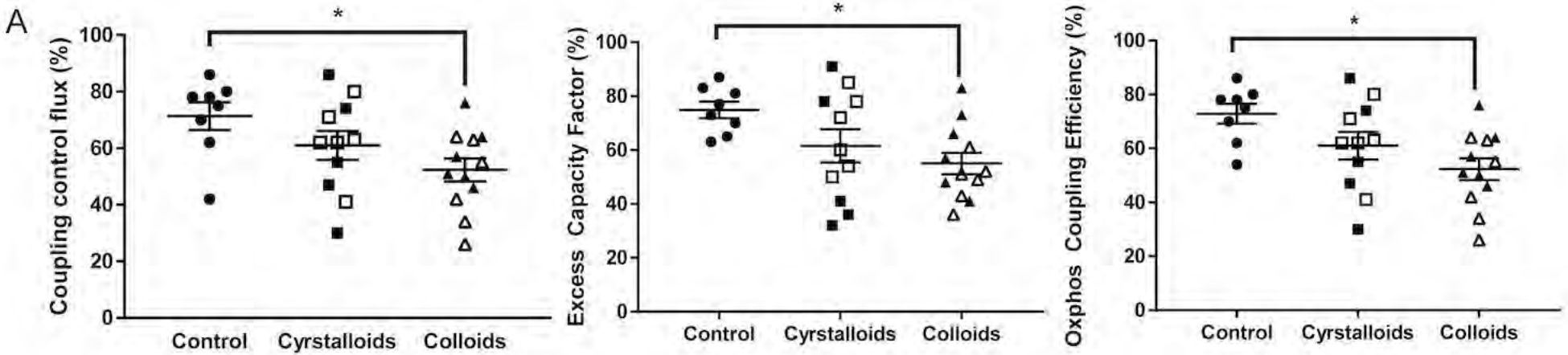
		24h	48h
Control (n=4)	Heart rate (% of BL)	102 ± 12	88 ± 9
	Mean arterial pressure (% of BL)	99 ± 25	82 ± 6
Burn-crystalloids (n=11)	Heart rate (% of BL)	105 ± 7	114 ± 7
	Mean arterial pressure (% of BL)	89 ± 8	88 ± 10
Burn-colloids (n=12)	Heart rate (% of BL)	101 ± 8	108 ± 6
	Mean arterial pressure (% of BL)	101 ± 8	100 ± 6

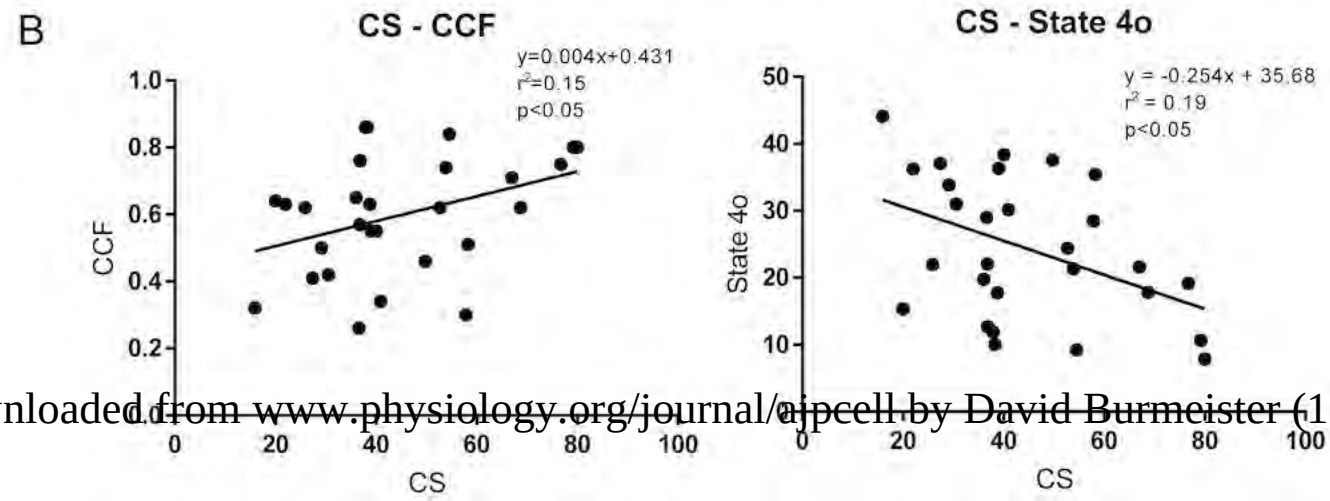
Values are mean ± SE

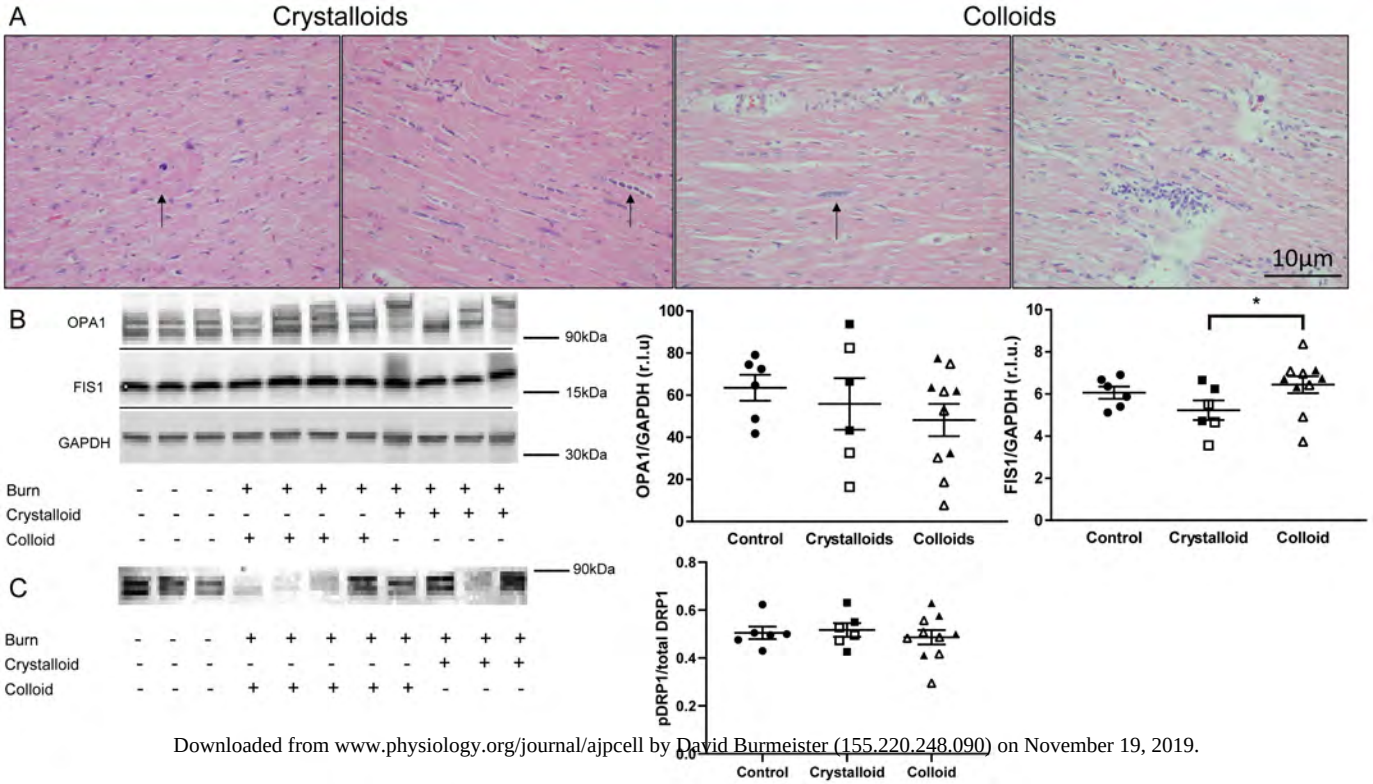


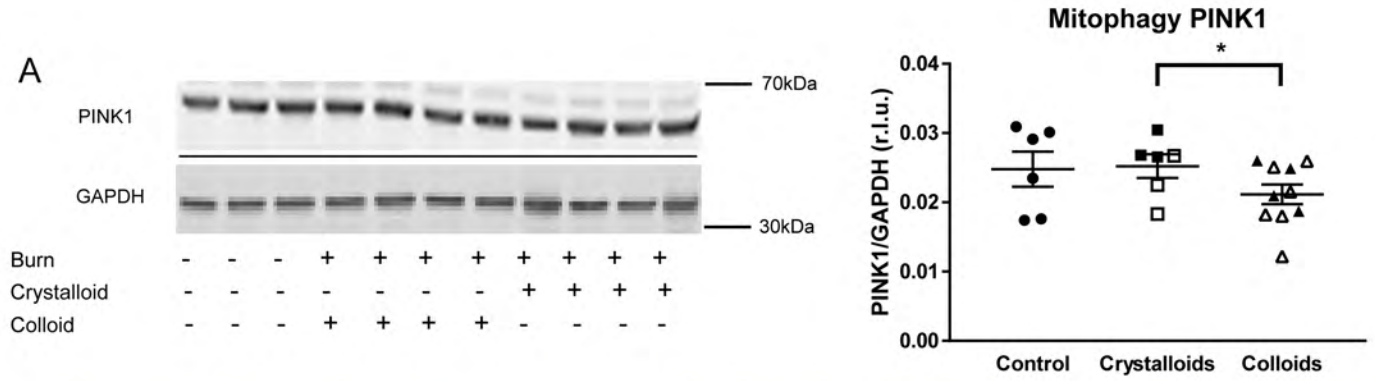


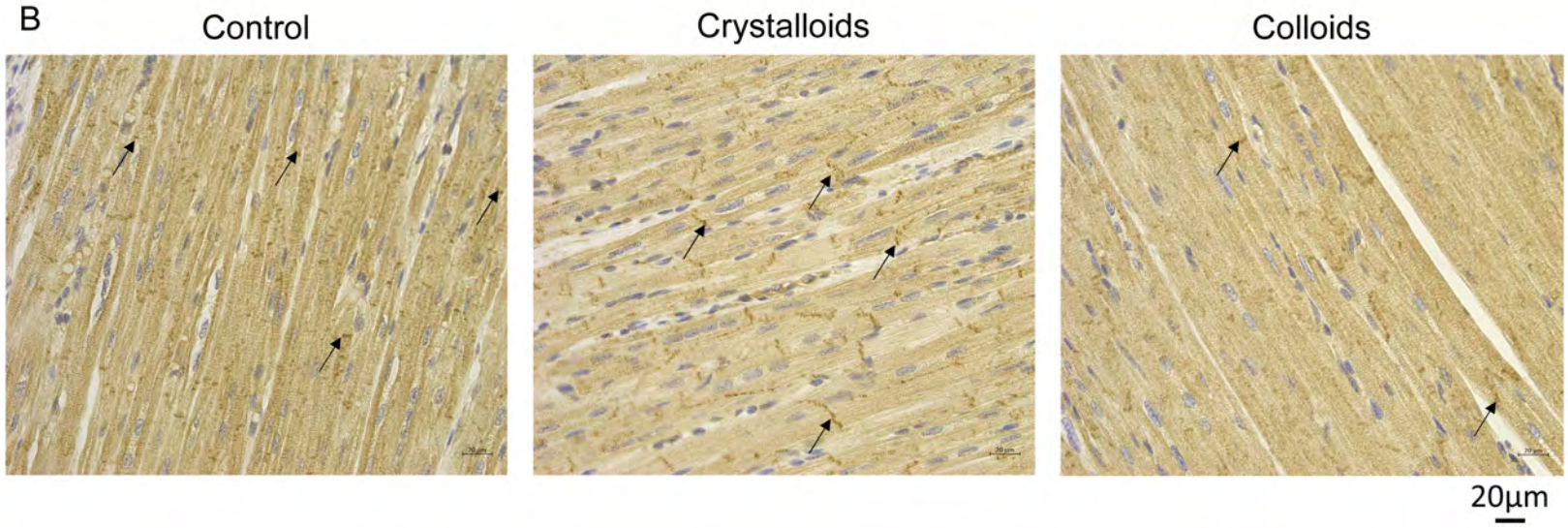












Effect of Intravenous Fluid Volumes on the Adrenal Glucocorticoid Response After Burn Injury in Swine

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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 (*3β-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* ≤ .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-1β, 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 underresuscitated, organ perfusion is not adequately maintained which leads to significant morbidity and mortality.

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Conflict of interest statement. None declared.
This study was supported by Congressionally Directed Medical Research Program Award# W81XWH-16-2-0041.
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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Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site.

doi:10.1093/jbcr/iry024

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

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 proteolysis[17] 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 PCO_2 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 ± 0.2°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 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 µm 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, fasiculata/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×*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×*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 RT^2 First Strand Kit from (Qiagen, Hilden, Germany). Prepared cDNA samples were diluted in SYBR (Bio-Rad Laboratories, Hercules, CA) with previously published primers[24–26] 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 (G*apdh*). 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, fasiculata/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 ± 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 ± 0.3°C) and MB (39.7 ± 0.03°C) when compared with the S-FR animals (38.5 ± 0.2°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 ± 10.73 mg/dl; $P < .001$) and 48 hours (189.00 ± 31.85 mg/dl; $P < .001$) when compared with BL levels (70.67 ± 6.58 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*), 3β-Hydroxysteroid dehydrogenase (*3β-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

	Temperature			Heart Rate			Respiratory Rate			Glucose		
Timepoint	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 fasiculata/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

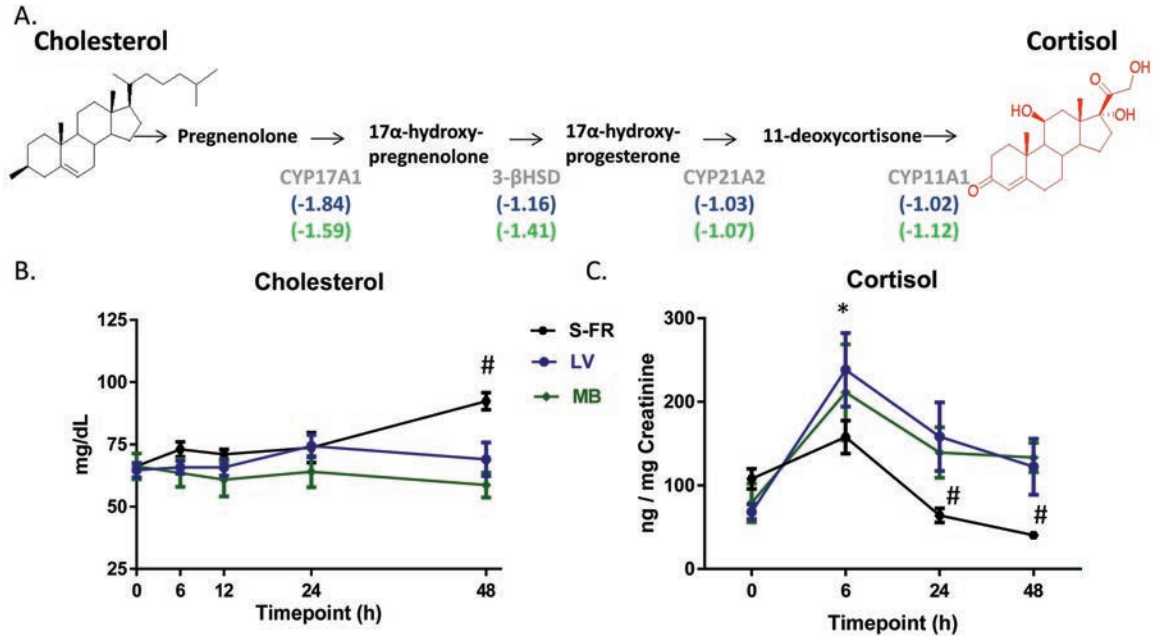

Figure 1. Diagram of cholesterol to cortisol synthesis and the steroidogenic enzymes (gray) required for conversion (A). Fold Regulation (blue LV and green MB) of steroidogenic enzymes cytochrome P450 family 11 subfamily A member 1 (*CYP11a1*), Cytochrome P450 Family 17 Subfamily A Member 1 (*CYP17a1*), 3β-Hydroxysteroid dehydrogenase (*3β-HSD*), Cytochrome P450 Family 21 Subfamily A Member 2 (C*YP21a2*). 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 ± 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.

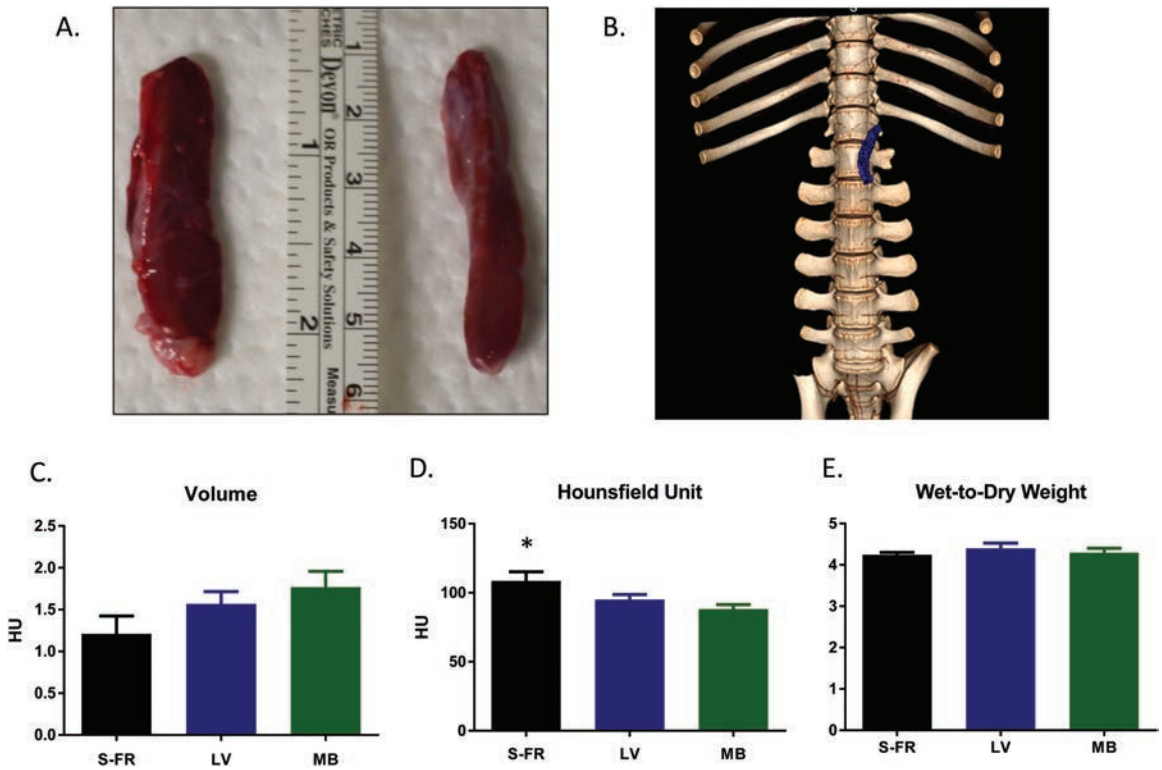

Figure 2. (A) Adrenal glands isolated immediately after euthanasia demonstrated no gross abnormalities between animals (representative picture shown is from MB group). (B) Computed tomography (CT) scanning was performed pre-injury and immediately prior to euthanasia (termination of experiment 48 hours; 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 ± SEM and * indicates a significant ($P < .05$) treatment difference.

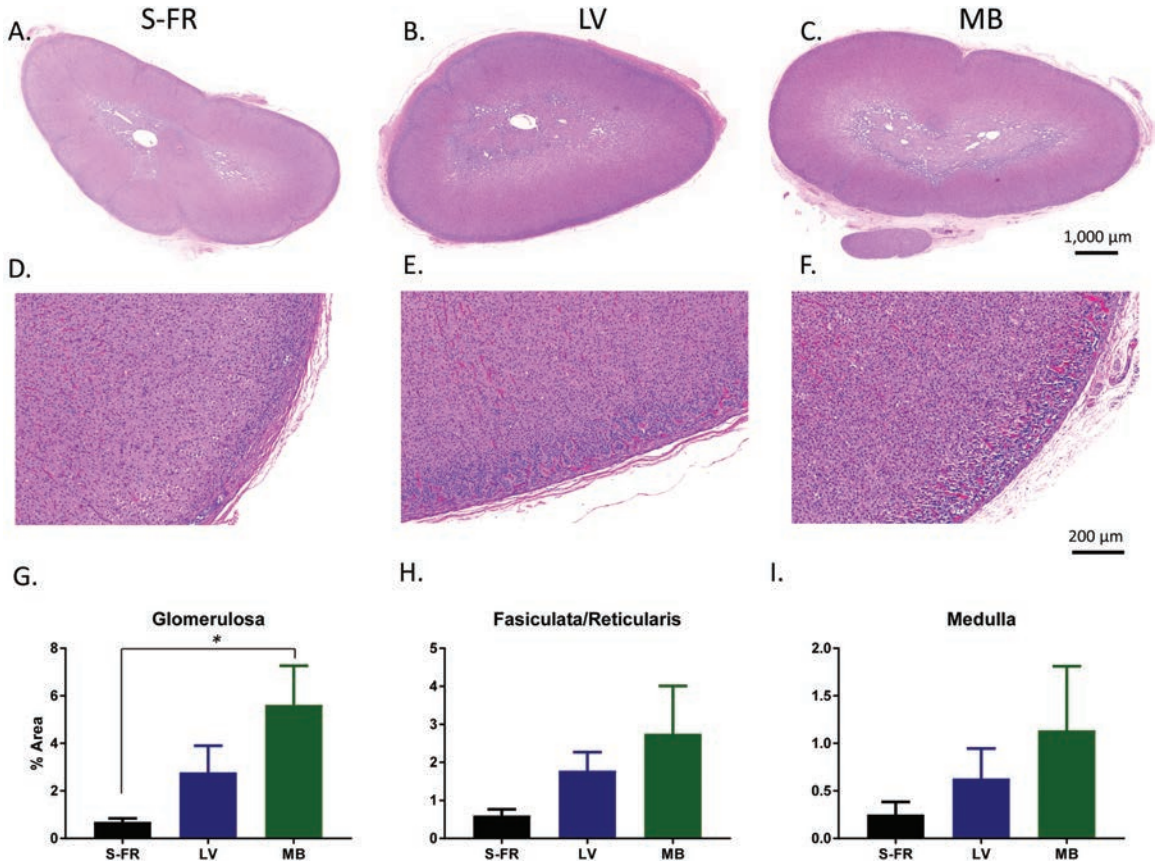

Figure 3. Representative hematoxylin and eosin (H&E) staining of the adrenal in S-FR (A and D), LV (B and E), and MB (C and F) animals. Images demonstrate hemorrhaging which are graphically represented as mean ± SEM of % surface area in the glomerulosa (G), fasiculata/reticularis (H), and medulla (I). S-FR ($n = 3$), LV ($n = 4$), MB ($n = 4$). Scale bars represent 1 mm for A–C, and 200 µm 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 IL1ra ($P < .05$) 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-1β 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

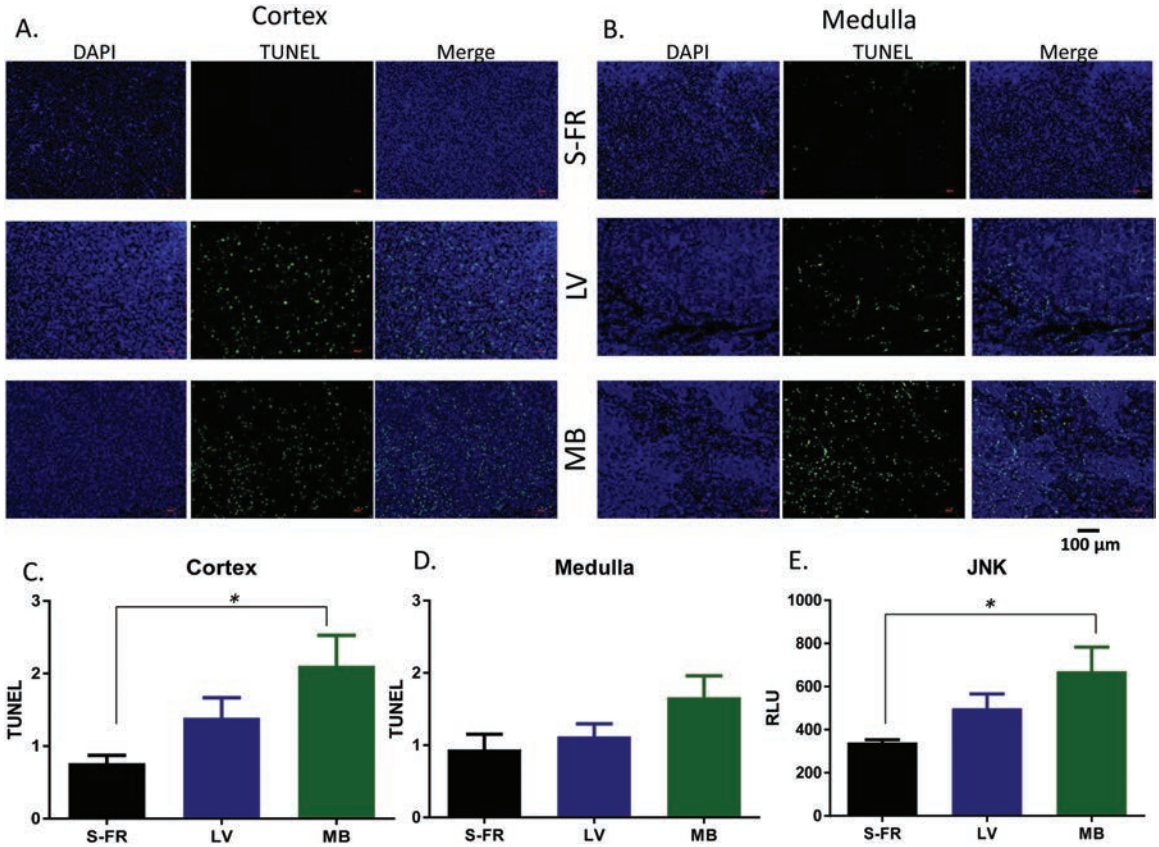

Figure 4. Representative adrenal TUNEL- and DAPI-stained adrenal cortex (A) and medulla (B) sections shows staining intensity and thus apoptosis between S-FR, LV, and MOB treatments. (C) Quantification of color density reveals significantly greater apoptosis in the cortex and medulla (D) of MB-treated 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 ± 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 µm. *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

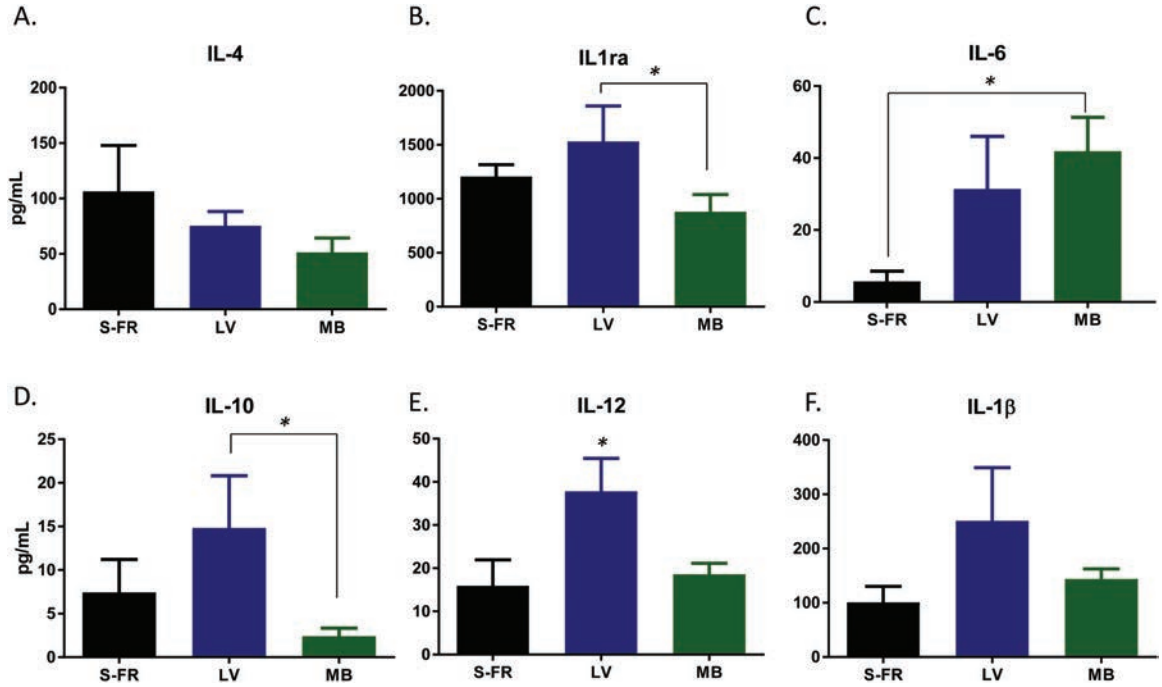

Figure 5. Cytokines IL-4 (A), IL1ra (B), IL-6 (C), IL-10 (D), IL-12 (E), and IL-1β (F) were quantified in adrenal tissue and values are presented as mean ± SEM of the observed concentration and * indicates a significant ($P < .05$) treatment difference. S-FR ($n = 3$), LV ($n = 4$), MB ($n = 5$). *LV*,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

RESEARCH ARTICLE

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

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Citation: Gómez BI, McIntyre MK, Gurney JM, Chung KK, Cancio LC, Dubick MA, et al. (2018) Enteral resuscitation with oral rehydration solution to reduce acute kidney injury in burn victims: Evidence from a porcine model. PLoS ONE 13(5): e0195615. https://doi.org/10.1371/journal.pone.0195615

Editor: Nick Ashton, The University of Manchester, UNITED KINGDOM

Received: November 21, 2017

Accepted: March 26, 2018

Published: May 2, 2018

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: CDMRP (http://cdmrp.army.mil/) award #W81XWH-16-2-0041 provided funding for this project. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

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/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±54mL/kg versus 120±24mL/kg, respectively), ORS intake increased urine output compared with water and no water (47.3 ±9.0 mL/kg versus 16.1±2.5 mL/kg, and 24.5±1.7 mL/kg respectively). Plasma creatinine peaked 6h following burn (1.67±0.07mg/dL) in all animals, but at 48h was comparable to BL in animals receiving water (1.23±0.06mg/dL) and ORS (1.30±0.09mg/dL), but not fluid deprived animals (1.56±0.05mg/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±0.17mm), whereas resuscitation with water (-0.44±0.14 mm) or ORS maintained it (-0.63±0.20 mm;P< 0.02). Circulating cytokines IL-1β, IL-6, IFNγ, 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 60ÊCoven 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 10mL/kg, a peak inspiratory pressure of 20 cm H_2O, and respiratory rate of 8 to 10 breaths/min. The ventilator was adjusted to achieve an end-tidal PCO_2 of 40±5 mm Hg. Animals were maintained on 1% to 3% isoflurane, balance O_2 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±0.2ÊCby 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-μm 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 K_2 EDTA-containing tubes and centrifuged at 4,300 x g for 10 min, aliquoted, and stored at -80ÊC 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 K_2 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: $((Creatinine_{urine} \times Volume_{urine})/ (Creatinine_{plasma}) \times Time_{min})$. 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 ±0.09ÊC),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 (BEecf) 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 ± 947 mL (132±54 mL/kg) versus 4,812 ± 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 ± 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.

	WBC ($1x10^3/\mu L$)			pH			Glucose (mg/dL)			BEecf (mmol/L)		
Time Point	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	ŧ7.49±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.

https://doi.org/10.1371/journal.pone.0195615.t001

±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

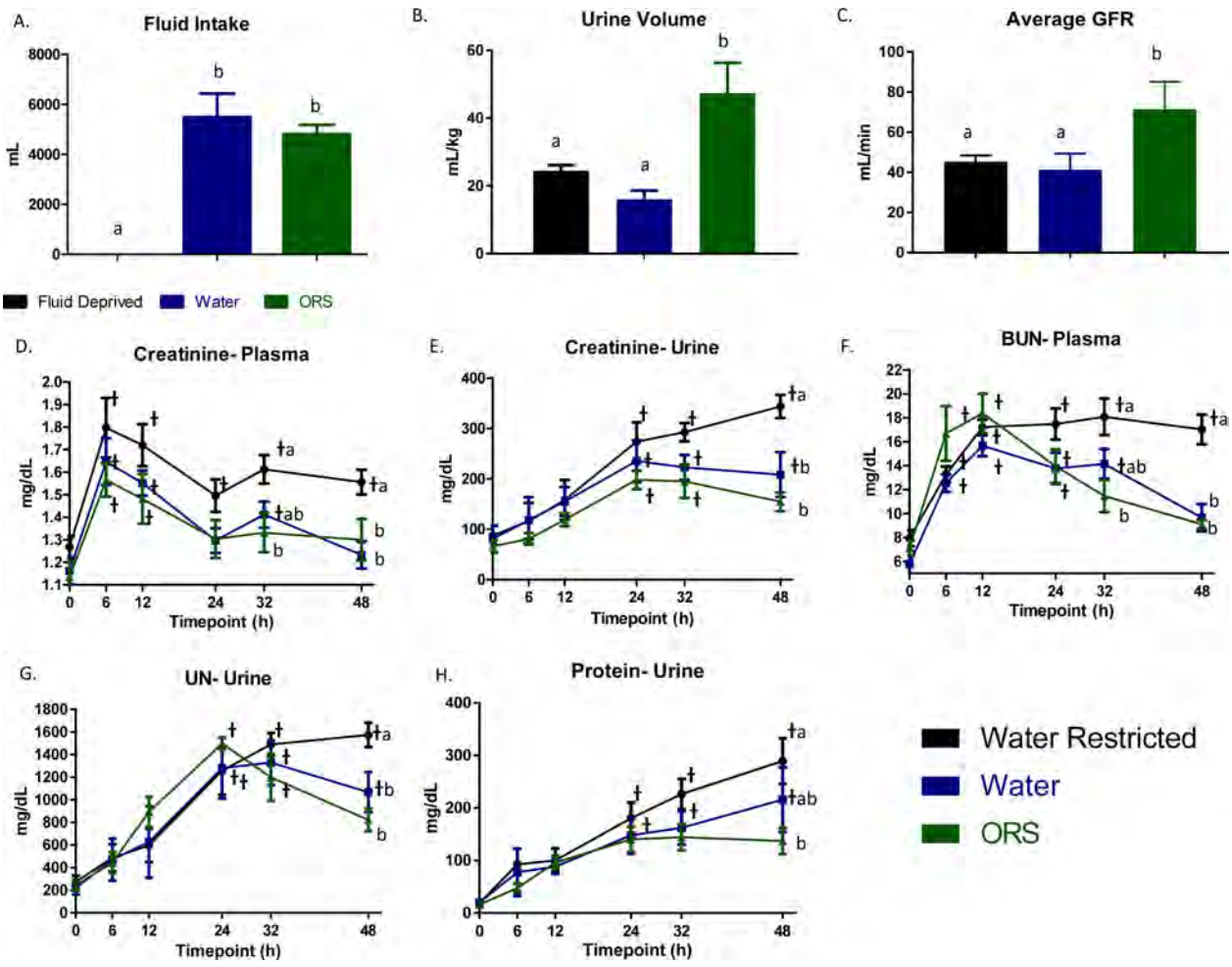

Fig 1. ORS increases urine output and positively alters burn-induced biochemical markers. Total fluid intake (A) urine output volume (B), and (C) Average glomerular filtration rate (GFR) throughout the duration of the study. Levels of creatinine (D, E) and urea nitrogen in the plasma (F) and the urine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

https://doi.org/10.1371/journal.pone.0195615.g001

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 cm^3), but did not change with fluid deprivation or animals consuming ORS groups (-0.38±5.2 and 1.2±5.5 cm^3, 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 glomerulonephrirtis hallmarked by microthrombi formation, synechia and parietal cell hypertrophy (Fig 3). Moderate to severe

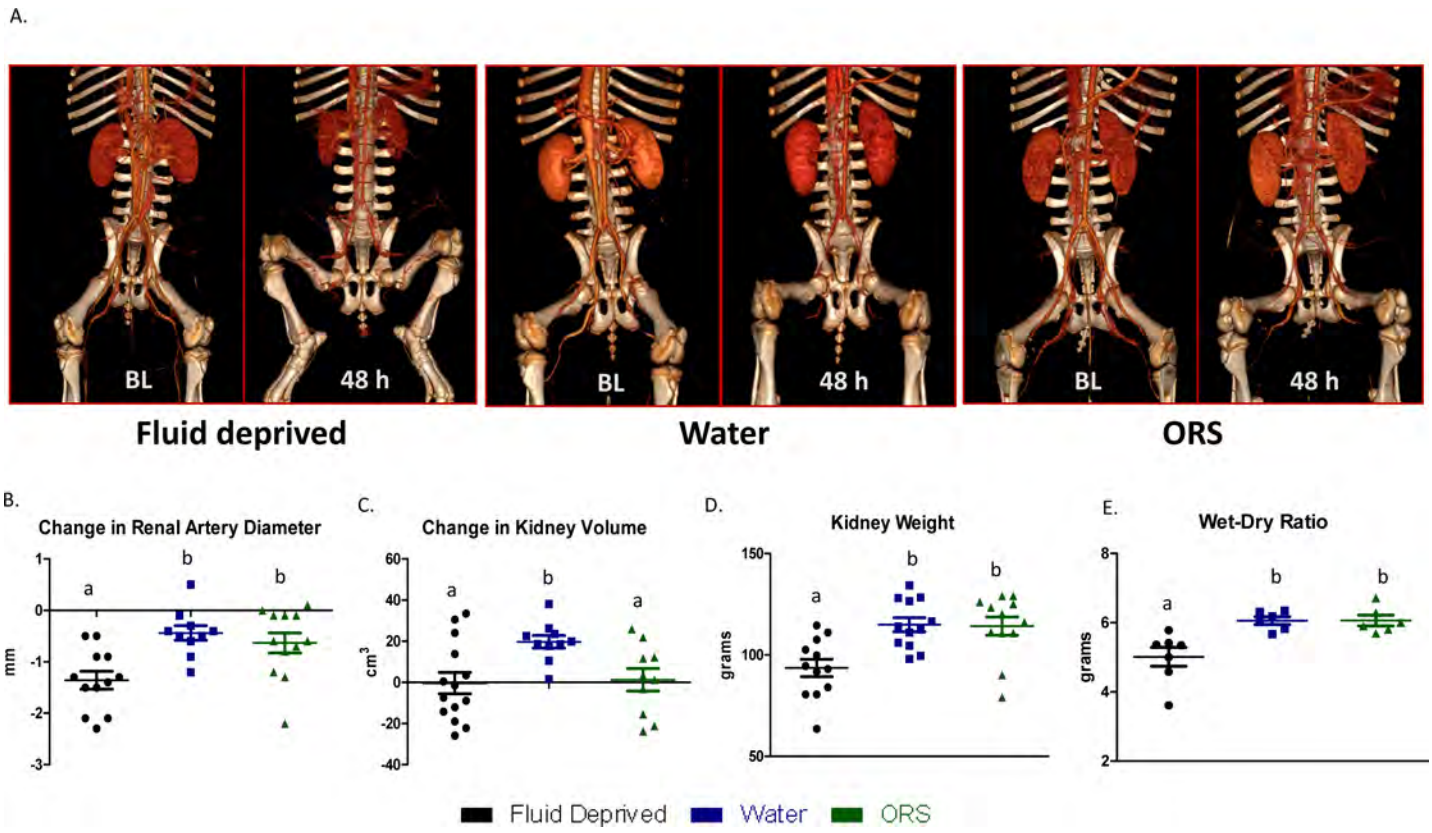

Fig 2. Enteral resuscitation prevents reduction in renal artery diameter. Computed tomography (CT) scanning (A) was performed pre-injury and immediately prior to euthanasia (termination of experiment 48 h). Renal artery diameter (B), kidney volume (C), weight (D), and wet:dry ratios (E) were quantified.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

https://doi.org/10.1371/journal.pone.0195615.g002

glomereulonephritis 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 ± 3.8mg/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-1β ($P \leq 0.019$), IL-6 ($P \leq 0.027$), and IFNγ ($P \leq 0.12$) 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-1ra 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

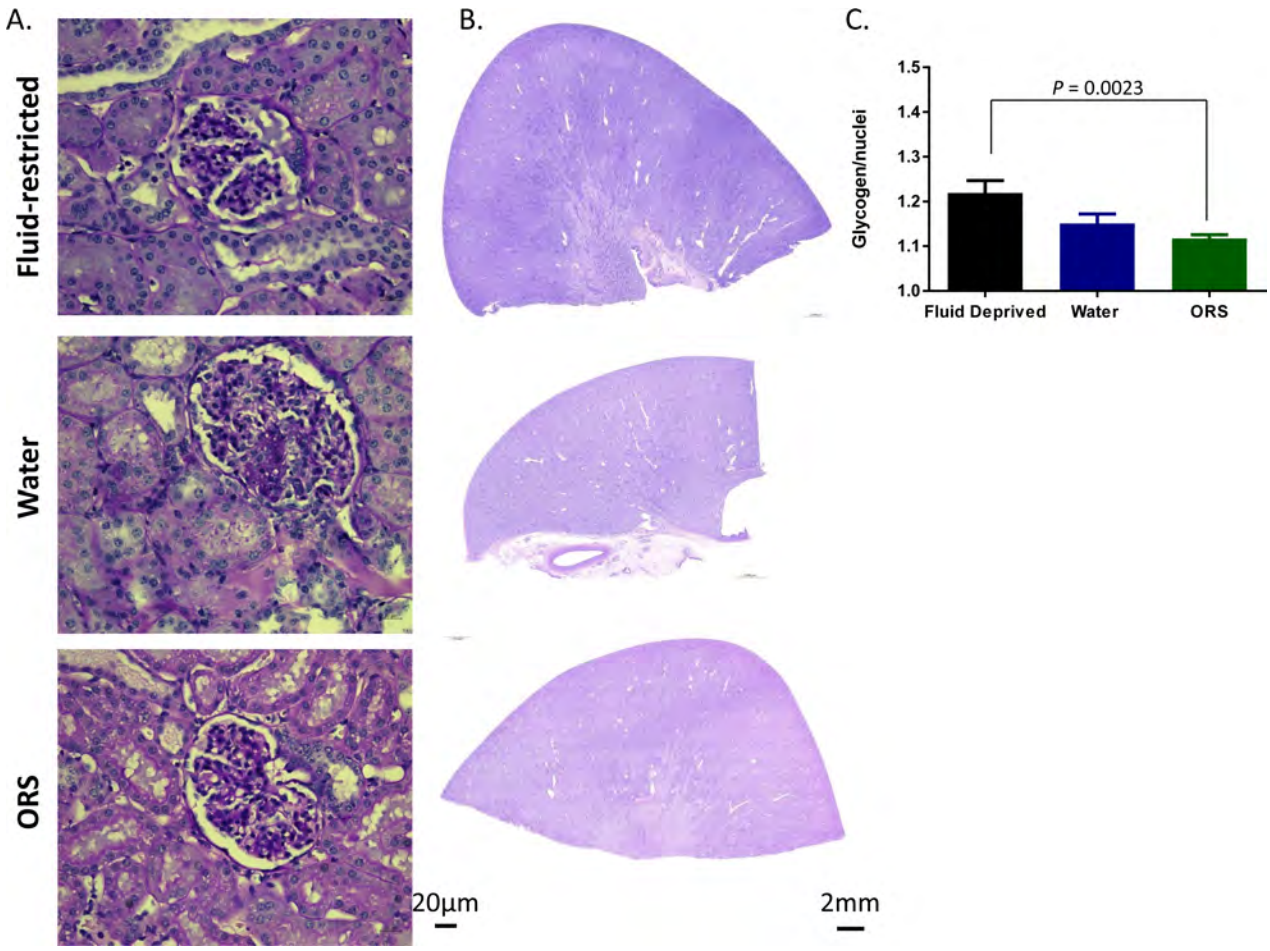

Fig 3. Renal glycogen content is greatest with fluid deprivation. (A) Representative H&E staining reveals hallmarks of glomerulonephririts in all groups, to include inflammatory cells (#), clotting with cell debris (*), parietal cell hypertrophy (arrow), and synechia (arrowhead) in all groups. Representation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

https://doi.org/10.1371/journal.pone.0195615.g003

($P < 0.05$; 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

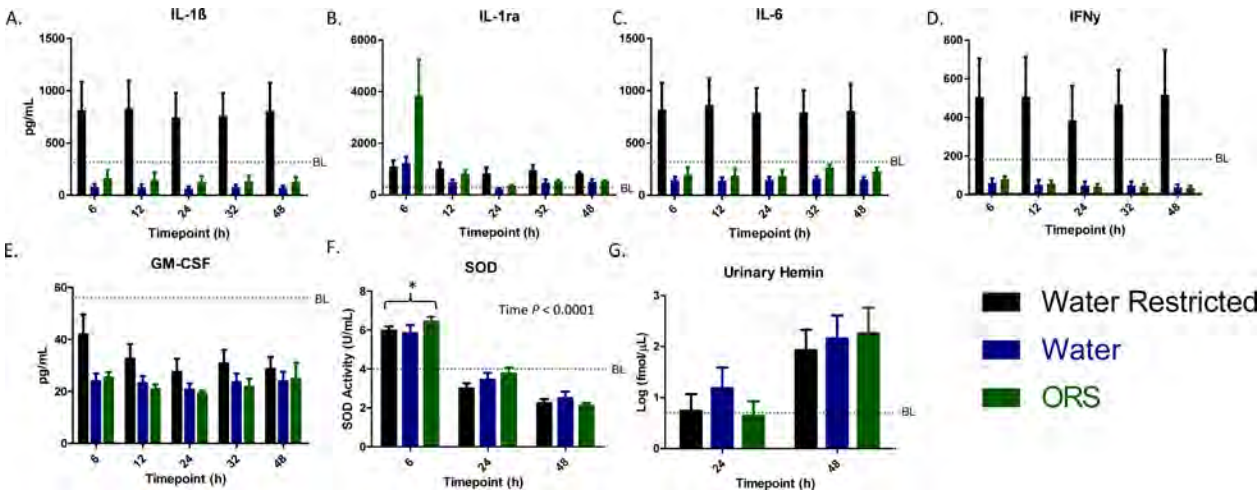

Fig 4. Fluid deprivation elevates circulating cytokines. Select circulating cytokines were quantified across all time points (A-E). Average of baseline values of all treatments is set as a reference line and bars represent mean ± SEM. Superoxide dismutase (SOD) activity in the plasma (F) and urinary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

https://doi.org/10.1371/journal.pone.0195615.g004

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 (Ceralyte®) 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 ± SEM with a different superscript letter are significantly different ($P < 0.05$) between treatments for indicated time point and a ɪ indicates a significant ($P < 0.05$) difference from the BL value.
(TIF)

Acknowledgments

The authors would like to thank the USAISR Veterinary and Laboratory Support Branches for their assistance.

Disclaimer: 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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BBA - Molecular Basis of Disease 1863 (2017) 2661–2671

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

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

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ARTICLE INFO

Keywords:
Acute kidney injury
Animal models
Hemorrhage
Burn
Inflammation
Oxidative stress

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., cisplatin), 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 multifactorial 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; NFκB, 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, Iκβ 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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http://dx.doi.org/10.1016/j.bbadis.2017.04.011
Received 22 November 2016; Received in revised form 14 February 2017; Accepted 10 April 2017
Available online 19 April 2017
0925-4439/ Published by Elsevier B.V.

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

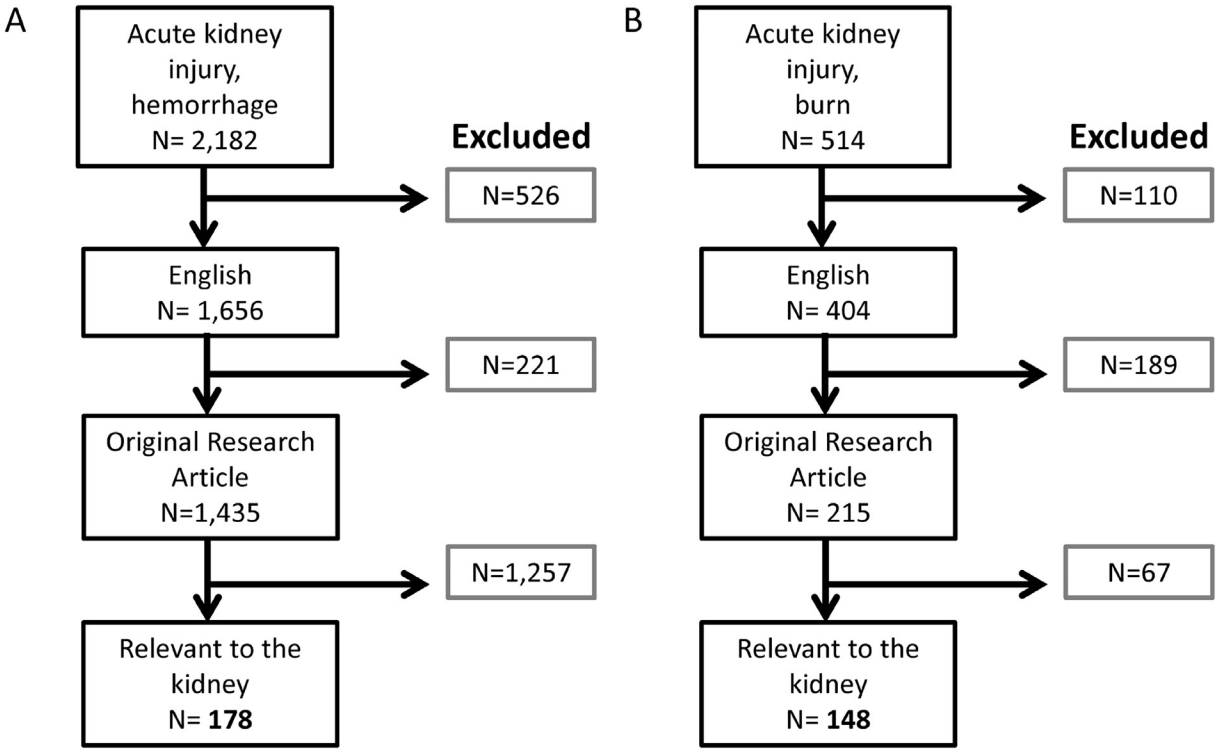

Fig. 1. Criteria for articles included in review from two individual PubMed literature searches using the key words: A) "acute kidney injury, hemorrhage" for retrieval of articles for hemorrhagic shock and B) "acute kidney injury, burn" for articles on burn injury.

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

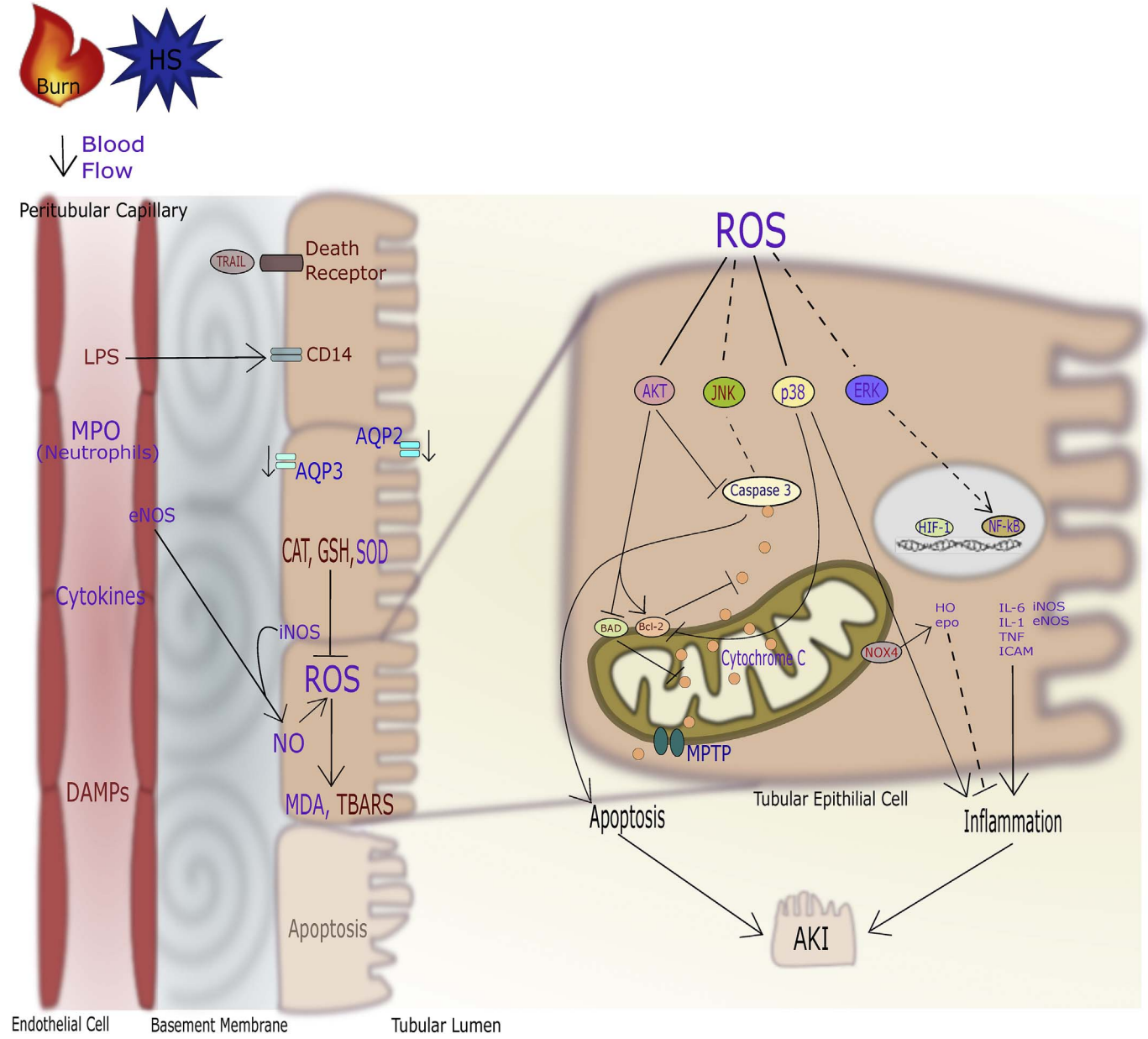

Fig. 2. Burn or hemorrhagic shock (HS) both result in reduced renal perfusion [17,18,60] that generates inflammatory and metabolic alterations in the tubule epithelia associated with acute kidney injury (AKI). Traumatic injury elicits an associated accumulation of reactive oxygen species (ROS) that result 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 (NFκB) 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, NFκB 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-κB) from the cytoplasm to the nucleus, which elicits the inflammatory cascade. Once in the nucleus, NF-κB 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, gingko 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-αR1 [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 hemoadsorption in a swine burn and smoke inhalation model [101]. In that study, IL-1β, 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 hemoadsorption 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 mitochondria-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-1β, 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 ulinistatin. 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 NFκB, 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-κB signaling pathways by inhibiting the IκB 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 IκBα thereby facilitating the release and translocation of NF-κB 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-1β, 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-1β 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.

Transparency document

The http://dx.doi.org/10.1016/j.bbadis.2017.04.011 associated with this article can be found, in online version.

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