The Role of Acetazolamide in Mitigating Inflammation and Innate Immune Activation at High Altitude

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Title of Project: The role of acetazolamide in mitigating innate immune activation at high altitude: a randomized cross-over controlled trial

Executive Summary: High altitude exposure can lead to an acute pro-inflammatory response and activation of innate immune cells.[1–6] Our prior work demonstrates that 1 to 3 days at 3800 m elevation leads to increased expression of inflammatory damage associated molecular patterns (DAMPs), several toll-like receptor pathways involved in innate immune responses, as well as increases in pro-inflammatory monocyte and neutrophil subpopulations.[1,6,7] Furthermore, our data and others indicate that either hypoxia-induced inflammatory responses, or baseline immune parameters, likely play a role in the onset and severity of Acute Mountain Sickness (AMS), although the precise mechanism is unknown.[7] One of the most effective medical treatments for AMS is Acetazolamide (AZ).[8] The mechanism of action by which AZ alleviates AMS is through regulation of blood bicarbonate levels, modification of blood pH, and resulting increases in ventilation and improved tissue oxygenation.[8–10] However, AZ is also reported to have anti-inflammatory and immune-modulatory properties in animal models, which have not been evaluated as a potential mechanism of action in AMS treatment.[11,12] We hypothesize that AZ will reduce pro-inflammatory responses and innate immune activation during 3 days of high-altitude exposure. To test this hypothesis, we will conduct a randomized, cross-over, placebo-controlled trial in which we administer AZ or placebo treatments to N=20 individuals over two visits to 3800 m elevation. Outcome measures will include the most detailed evaluation of systemic inflammatory and immune parameters to date in this field, as well as contributing physiological parameters including ventilatory chemoreflex sensitivity, and daytime and nocturnal pulse oximetry. This work will advance our knowledge about the pathology and treatment of a key wilderness medicine challenge by identifying a potential novel mechanism of AMS onset and treatment.

Section I – Summary

A. Introduction and Background: Acetazolamide (AZ) is a highly effective treatment for acute mountain sickness (AMS) at high altitude.[8,9] AZ mitigates AMS through its activity as a renal carbonic anhydrase inhibitor, which decreases blood pH and stimulates ventilation to improve oxygen delivery to tissue.[9,10] However, AZ also demonstrates immune modulatory properties, with most evidence examining this in lung tissue to investigate the impact on pulmonary hypertension in animal models.[11,13] Our group and others show that high-altitude exposure results in an acute pro-inflammatory response and activation of the innate immune system, which some predict contributes to the onset and severity of AMS.[1,7] Therefore, the anti-inflammatory properties of AZ may play a key role in modulating AMS symptoms independent of ventilatory stimulation. However, prior work has examined the impact of AZ on only a select few inflammatory mediators, and the impact of AZ on immune function has never been evaluated.

B. Hypothesis and specific aims: Our goal is to determine if AZ mitigates the pro-inflammatory innate immune response during high altitude travel. We hypothesize that AZ improves AMS symptoms, in part, by reducing systemic hypoxia-induced inflammation and reducing the activation of innate immune cells (monocytes and neutrophils). We will test this hypothesis with the following experimental aims: (**Aim 1**) Determine if AZ treatment during high altitude exposure blunts plasma inflammatory cytokine expression and levels of circulating pro-inflammatory innate immune cell subsets; (**Aim 2**) Determine if AZ treatment during high altitude exposure modulates immune cell function (innate inflammatory responses to bacterial and viral stimuli, cell migration, and bacterial killing efficacy).

C. Study design and methods:

a. Study Design: This is a double blind, randomized, cross-over, placebo-controlled trial. Participants (N=20) will be recruited by word of mouth and advertising, and complete written

informed consent in the UC Riverside laboratory within 1 month of first ascent (Summer 2025). Participants then complete medical history and basic physical screening. At this time, they are randomly assigned to a treatment group (*i.e.* Group A taking AZ on trip 1 and placebo on trip 2), maintaining age and sex ratios across groups. Participants will be asked to begin taking their assigned treatment as indicated 2 days before each ascent (125 mg twice daily for AZ).[14] On the morning of ascent, participants arrive at the lab at 6:00 am and fasting peripheral venous blood samples will be collected by a licensed phlebotomist or physician. Participants then travel to Barcroft Station (3800 m elevation) *via* vehicle (6 -7 hours). Over three days at high altitude, participants complete morning fasting blood draws, morning and evening AMS questionnaires (Lake Louise)[15], and morning and evening physiological measures (blood pressure, heart rate, SpO_2). On the second day at high altitude, ventilatory chemoreflex measures will be collected as previously described by our group.[16] WatchPAT sleep kits will be used to characterize nocturnal hypoxic burdens, oxygen desaturation indices, and apnea hypopnea indices as previously described by our group.[17] Following descent, participants complete 1-week and 1-month follow-up visits to determine the long-term effects of high-altitude exposure on immune phenotypes.

100 µl of whole blood will be fixed and stained immediately on-site at each timepoint for flow cytometry analysis at UC Riverside within 3 days of sample collection. We will also conduct complete blood counts on-site. 20 ml of EDTA-treated blood will be utilized for plasma collection and isolation of peripheral blood mononuclear cells by density gradient centrifugation. Isolated immune cells will be immediately frozen and stored in liquid nitrogen for transport to UC Riverside using a novel protocol optimized by our group for cryogenically storing viable cells in remote field locations.[7] Upon return to sea level, immune cells will be cultured in the presence or absence of several stimulants including bacterial lipopolysaccharide (LPS) and R848 (Resiquimod, stimulating the antiviral response). Following stimulation, we will collect culture

supernatants and measure expression of inflammatory factors using Legendplex multi-plex cytokine panels as previously described by our group.[18] These panels allow simultaneous measurement of many key inflammatory mediators including 13 inflammatory cytokines, 12 markers of vascular inflammation, and 13 anti-viral response factors. We will also conduct additional functional assays, including bacterial killing and cell migration assays. Plasma cytokine levels will also be measured via Legendplex. All cell culture assays will be conducted in triplicate with downstream cytokine profiling in duplicate.

b. Power calculations: Sample sizes are based on power calculations targeting 80% power to detect significant differences across sea level and high altitude at $\alpha = 0.05$. Calculations were performed using *pwr* in R and are based on previously published data or our own preliminary work. A sample size of 14 is required to detect a significant shift from classical to intermediate monocyte subsets after 1 day at high altitude with an effect size in our prior studies of 0.46.[7] Additionally, a sample size of 10-36 is required to detect significant correlations between AMS score and classical ($R^2 = 0.79$) or non-classical monocytes ($R^2 = 0.45$). Finally, we previously identified elevations in inflammatory response genes after 1-3 days at high altitude with a sample size of 17.[1] Thus, we predict we are well powered with a sample size of 20 individuals (cross-over design) to detect significant changes in immune phenotypes at high altitude as well as correlations between immune cell distributions and AMS scores. This sample size is also consistent with other studies examining the efficacy of AZ for AMS treatment.[19]

c. Data analysis plan: Treatments will be assigned by a third party and all study team members and participants will be blinded. Data analysis will be conducted by a research team member who is blinded to participant treatment, demographic information, and day of sample collection. All samples and physiological data will be deidentified and coded. To determine if AZ treatment is linked to reduced inflammatory cytokine expression and reduced levels of pro-inflammatory

innate immune cell subsets (Aim 1), we will first utilize two-way repeated measures ANOVA to compare plasma cytokine expression and cell distributions across study days and treatments. To determine the impact of AZ treatment on innate immune cell function (Aim 2), we will use a three-way ANOVA to examine cell cytokine secretion across study days, treatments, and pre/post cell stimulation. Significant main effects will be further evaluated with post-hoc Tukey's HSD tests, and we will verify our data meets all required test assumptions. We will carefully examine impacts of sex, age, BMI and other demographic factors on outcomes using mixed linear models. WatchPAT data will be analyzed according to the American Academy of Sleep Medicine criteria by a qualified sleep technician and ventilatory parameters will be analyzed as described previously.[16,20–22] To determine if these parameters are associated with our immune and AMS outcomes, we will conduct mixed linear models with trip, day, treatment, and sex, as covariates.

d. Timeframe: This study will take place in the summer of 2025. Participants will be recruited in the month prior to study start, and work will be conducted over two expeditions. Participants will complete baseline testing within one week of ascent. They will then arrive on the morning of ascent for sea-level blood sampling, be driven to Barcroft Station over 6 hours in vans, and will remain at the station for 3 nights (4 days) during each visit. The group will then return to sea level and a follow-up appointment will be completed 1 week and 1 month following return. The second trip will take place 1-2 months after the first. Flow cytometry and multiplex cytokine panel tests will be conducted immediately after each trip, and cell functional assays will be completed within 2 months of return. Final data analyses will be complete over the following 2 months in time for an abstract submission to the American Physiology Summit by the deadline in Fall 2025 and attendance of the 2026 Winter Conference of the Wilderness Medical Society. The manuscript resulting from this work will be submitted for publication in *Wilderness & Environmental Medicine* by Summer 2026.

e. Facilities: This study will be performed at the UC Riverside School of Medicine and Barcroft Station in the White Mountain Research Center. Barcroft Station is located approximately 6 hours northeast of UC Riverside and our team has extensive experience at this site. The facilities house up to 20 participants in dormitory-style lodging, provides three meals per day, and contains separate laboratory spaces in for blood sample processing and cell collection. Our facilities at UC Riverside include a large, biosafety level 2 laboratory space with separate space for studies with human subjects. We have access to all necessary equipment to this work including a NovoCyte Quanteon Flow Cytometer, biosafety cabinets and incubators, centrifuges, fridges and freezers, and cryogenic storage for cells.

D. Institutional Review Board approval: Approval for this study has been granted by the UC Riverside IRB (project #HS-22-088). An amendment will be required to add the AZ treatment, and will be submitted for approval immediately following notice, if we are funded.

E. Clinical Trial Registration: This study will be registered at ClinicalTrials.gov prior to the start of data collection and recruitment. We will work with our UC Riverside Clinical Trial Coordinator, Herlinda Bergman, to verify we meet all regulatory requirements.

F. Significance: This work aligns with the mission of WMS to improve our scientific knowledge of human health activities in a wilderness environment. It will provide a novel mechanism by which AZ mitigates AMS and may lead to new therapeutic approaches. Furthermore, we will generate new knowledge regarding the precise ways in which high altitude exposure influences immune function which has implications for understanding the impact of hypoxemia and physiological stress on infectious disease susceptibility. This work may also indicate a novel role for immune activation in AMS development.

Section II – References

1. Pham K, Frost S, Parikh K, Puvvula N, Oeung B, Heinrich EC. Inflammatory gene expression during acute high-altitude exposure. *The Journal of Physiology*. 2022;600(18):4169-4186. doi:10.1113/JP282772

2. Pham K, Parikh K, Heinrich EC. Hypoxia and Inflammation: Insights From High-Altitude Physiology. *Front Physiol*. 2021;12:676782. doi:10.3389/fphys.2021.676782

3. Facco M, Zilli C, Siviero M, et al. Modulation of Immune Response by the Acute and Chronic Exposure to High Altitude: *Medicine & Science in Sports & Exercise*. 2005;37(5):768-774. doi:10.1249/01.MSS.0000162688.54089.CE

4. Feuerecker M, Crucian BE, Quintens R, et al. Immune sensitization during 1 year in the Antarctic high-altitude Concordia Environment. *Allergy*. 2019;74(1):64-77. doi:10.1111/all.13545

5. Meehan R, Duncan U, Neale L, et al. Operation Everest II: Alterations in the immune system at high altitudes. *J Clin Immunol*. 1988;8(5):397-406. doi:10.1007/BF00917156

6. Pham K, Vargas A, Frost S, Shah S, Heinrich E. Changes in immune cell populations during acclimatization to high altitude. *Physiological Reports*. in press.

7. Vargas A, Mkrtchyan K, Penuelas V, et al. Acute Exposure to High-Altitude Results in Changes to Immune Cell Populations. *Physiology*. 2024;39(S1):2515. doi:10.1152/physiol.2024.39.S1.2515

8. Forwand SA, Landowne M, Follansbee JN, Hansen JE. Effect of Acetazolamide on Acute Mountain Sickness. *N Engl J Med*. 1968;279(16):839-845. doi:10.1056/NEJM196810172791601

9. Larson EB. Acute Mountain Sickness and Acetazolamide: Clinical Efficacy and Effect on Ventilation. *JAMA*. 1982;248(3):328. doi:10.1001/jama.1982.03330030034021

10. Leaf DE, Goldfarb DS. Mechanisms of action of acetazolamide in the prophylaxis and treatment of acute mountain sickness. *Journal of Applied Physiology*. 2007;102(4):1313-1322. doi:10.1152/japplphysiol.01572.2005

11. Michael Z, Christou H, Hudalla H, et al. Acetazolamide Modulates Pulmonary Inflammation and Ameliorates Severe Experimental Pulmonary Hypertension. *Pediatrics*. 2019;144(2_MeetingAbstract):695-695. doi:10.1542/peds.144.2MA7.695

12. Wang C, Wang R, Xie H, et al. Effect of acetazolamide on cytokines in rats exposed to high altitude. *Cytokine*. 2016;83:110-117. doi:10.1016/j.cyto.2016.04.003

13. Hudalla H, Michael Z, Christodoulou N, et al. Carbonic Anhydrase Inhibition Ameliorates Inflammation and Experimental Pulmonary Hypertension. *Am J Respir Cell Mol Biol*. 2019;61(4):512-524. doi:10.1165/rcmb.2018-0232OC

14. Hackett P, Shilm D. CDC Yellow Book 2024: High Elevation Travel & Altitude Illness. https://wwwnc.cdc.gov/travel/yellowbook/2024/environmental-hazards-risks/high-elevation-travel-and-altitude-illness

15. Roach RC, Hackett PH, Oelz O, et al. The 2018 Lake Louise Acute Mountain Sickness Score. *High Altitude Medicine & Biology*. 2018;19(1):4-6. doi:10.1089/ham.2017.0164

16. Frost S, Pham K, Puvvula N, Oeung B, Heinrich EC. Changes in hypoxic and hypercapnic ventilatory responses at high altitude measured using rebreathing methods. *Journal of Applied Physiology*. 2024;137(2):364-373. doi:10.1152/japplphysiol.00128.2024

17. Frost S, Orr J, Oeung B, et al. Improvements in sleep-disordered breathing during acclimatization to 3800 m and the impact on cognitive function. *Physiol Rep*. 2021;9(9). doi:10.14814/phy2.14827

18. Bergersen KV, Pham K, Li J, et al. *Health Disparities in COVID-19: Immune and Vascular Changes Are Linked to Disease Severity and Persist in a High-Risk Population in Riverside County, California*. In Review; 2023. doi:10.21203/rs.3.rs-2800664/v1

19. Gao D, Wang Y, Zhang R, Zhang Y. Efficacy of Acetazolamide for the Prophylaxis of Acute Mountain Sickness: A Systematic Review, Meta-Analysis and Trial Sequential Analysis of Randomized Clinical Trials. *The American Journal of the Medical Sciences*. 2021;361(5):635-645. doi:10.1016/j.amjms.2020.12.022

20. Duffin J. Measuring the respiratory chemoreflexes in humans. *Respiratory Physiology & Neurobiology*. 2011;177(2):71-79. doi:10.1016/j.resp.2011.04.009

21. Duffin J. Measuring the ventilatory response to hypoxia: Measuring ventilatory response to hypoxia. *The Journal of Physiology*. 2007;584(1):285-293. doi:10.1113/jphysiol.2007.138883

22. *AASM Manual for the Scoring of Sleep and Associated Events: Rules, Terminology and Technical Specifications*. Vol Version 3. American Academy of Sleep Medicine; 2023.