Preventing High Altitude Cerebral Edema in Rats with Repurposed Anti-Angiogenesis Pharmacotherapy

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BACKGROUND: High altitude cerebral edema (HACE) is a fulminant, deadly, and yet still unpredictable brain disease. A new prophylactic treatment for HACE and its predecessor, acute mountain sickness (AMS), needs to be developed without the contraindications or adverse effect profiles of acetazolamide and dexamethasone. Since neovascularization signals are likely key contributors to HACE/AMS, our approach was to examine already existing anti-angiogenic drugs to inhibit potential initiating HACE pathway(s). This approach can also reveal crucial early steps in the frequently debated mechanism of HACE/AMS pathogenesis.

METHODS: We exposed four rat cohorts to hypobaric hypoxia and one to sea level (hyperbaric) conditions. The cohorts were treated with saline controls, an anti-angiogenesis drug (motesanib), a pro-angiogenesis drug (deferoxamine), or an intraperitoneal version of the established AMS prophylaxis drug, acetazolamide (benzolamide). Brain tissue was analyzed for cerebrovascular leak using the Evans Blue Dye (EVBD) protocol.

RESULTS: We observed significantly increased EVBD in the altitude control and pro-angiogenesis (deferoxamine) cohorts, and significantly decreased EVBD in the anti-angiogenesis (motesanib), established treatment (benzolamide), and sea-level cohorts.

DISCUSSION: Anti-angiogenesis-treated cohorts demonstrated less cerebrovascular extravasation than the altitude control and pro-angiogenesis treated rats, suggesting promise as an alternative prophylactic HACE/AMS treatment. The leak exacerbation with pro-angiogenesis treatment and improvement with anti-angiogenesis treatment support the hypothesis of early neovascularization signals provoking HACE. We demonstrate statistically significant evidence to guide further investigation for VEGF- and HIF-inhibitors as HACE/AMS prophylaxis, and as elucidators of still unknown HACE pathogenesis.

KEYWORDS: HACE prophylaxis, acute mountain sickness, altitude sickness, vasogenic edema, cerebrovascular leak, blood brain barrier leak, neovascularization.

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Since 1980 we have made little progress preventing deaths on mountains such as Everest,[13] many of which are directly attributed to high altitude cerebral edema (HACE) and indirectly to its predecessor, acute mountain sickness (AMS).[5] Incidence rates of AMS and HACE appear to be increasing, with recent studies of Qinghai-Tibetan railway workers reporting 45–95% and 0.5%, respectively,[16] and overall HACE risk ranging up to 2%.[5] The FDA approval of acetazolamide for AMS prophylaxis has since been accompanied by significant controlled,[8,9] but retrospectively inept symptomatic effects.[4] Acetazolamide's common adverse effects include polyuria (55% incidence at 250 mg) and taste disturbance (11% incidence at 500 mg), which may improve with decreased dose, but also significant paresthesia (68% incidence at 250 mg), which was not found to decline with decreased dose.[9] Furthermore, patients unlikely to benefit from acetazolamide include those sensitive to sulfonamides, those with hepatic

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or renal impairment, pregnant patients, and those taking cardiac glycosides, antihypertensives, lithium, or high dose aspirin.[8]

Acetazolamide works by decreasing proximal tubule sodium and bicarbonate reabsorption, countering the alkalosis-induced hypoventilatory response characteristic of altitude respiration, thereby allowing increased respiratory drive and oxygenation. However, acetazolamide is primarily a diuretic, from the carbonic anhydrase inhibitor class, and therefore carries increased risk of dehydration. Most high altitude climbers breathe dry cold air, with increased respiratory rates, greater fluid utilization through exercise, but typically decreased oral intake and fluid access, and often increased fluid loss via local diarrheal illness. Dehydration or physical exhaustion symptoms entirely overlap all AMS symptoms,[5,12] possibly causing inflated AMS diagnosis prevalence in a zebra-diagnosed-over-horse pattern. Probable hypovolemia and hemoconcentration, worsened by any diuretic, in turn lead to the likely increased incidence of deep vein thromboses and pulmonary emboli among mountaineers.[7]

Notably, the pathogenesis of HACE is still unknown. There are several main hypotheses, including: venous outflow obstruction (and/or increased arterial flow) leading to increased cerebral vessel size and leak,[1,15,16] cytotoxic edema due to reactive oxygen species (ROS),[14,16] and vessel fenestration as a predictable step in the vascular endothelial growth factor (VEGF) angiogenesis pathway.[2] The latter path follows: 1) hypoxia, causing cerebral cells to release 2) neovascularization signals, especially hypoxia-inducible factor (HIF-1A-1alpha) and VEGF, which 3) fenestrate existing vessels at anticipated branch points of new vessels.[3] This results in 4) vasogenic edema.[16]

Thus, a pathway meant to build long-term new vasculature to prevent future hypoxia may have pathological short-term consequences. Early angiogenesis, before new vessels are even formed, requires the risk of vascular permeability as a necessary precursor of new vascular branching.[3] In fact, the hypoxic signal molecule VEGF was previously known as the vascular permeability factor (VPF), and has been shown to cause 50,000 times more vascular leak than histamine.[3]

One approach that would simultaneously test for novel effective HACE prophylaxis, as well as further elucidate the unknown disease pathway, is to employ known path-specific treatments at altitude and assess their effects on measured disease outcome. Specifically a HIF- or VEGF-inhibitor, for instance, would be expected to prevent HACE, while a HIF- or VEGF-inducer would be expected to worsen HACE if the hypoxic vasogenic edema pathway is correct.

Pro- and anti-angiogenic drugs, specifically HIF and VEGF inducers and inhibitors, should be tested for efficacy in HACE/AMS prevention. This should both test for potential prophylactic benefit and reveal whether HIF-1α and VEGF are integral to HACE pathogenesis. Subjects should be exposed to high altitude conditions and objectively assessed for the defining endpoint of AMS/HACE, cerebral edema.

METHODS

Animals

All protocols were reviewed and approved by the University of Colorado's Institutional Animal Care and Use Committee (IACUC) prior to implementation. Rats were all 8- to 10-wk old (300 g) male Sprague Dawley rats (Charles River Laboratories, Inc., Wilmington, MA).

Equipment and Materials

Cerebral tissue was assessed with a spectrophotometer (Labsystems Multiscan, Helsinki, Finland) at 600 nm to identify extravasated Evans Blue dye. Drugs chosen include deferoxamine, a pro-angiogenic drug that stabilizes HIF-1α; motesanib diphosphate, an anti-angiogenic drug that inhibits VEGF; and benzolamide, an intraperitoneal carbonic anhydrase inhibitor chosen to represent the current established AMS treatment, acetazolamide. All drugs were solubilized under aseptic conditions in saline (deferoxamine) or DMSO (motesanib, benzolamide) and loaded in identical hygienic single use syringes.

Procedure

Upon arriving in Denver, 25 rats were initially housed in hyperbaric chambers simulating sea level to prevent acclimatization to Denver altitude. After 8 d the rats were randomly divided into five groups ($N = 5$ per group): sea level control, high altitude control, pro-angiogenesis altitude treatment, anti-angiogenesis altitude treatment, and established altitude treatment (**Table I**). The latter four groups were moved to hypobaric chambers to mimic altitude approximating 17,500 ft (5334 m; Table I).

All rats were then given daily 0.3-mL intraperitoneal injections, the composition of which was determined by group: 20 mg · kg^{-1} of deferoxamine, 100 mg · kg^{-1} of motesanib diphosphate, 10 mg · kg^{-1} of benzolamide, or 0.3 mL of saline for all controls (Table I). After 10 d of exposure with treatment, the rats were anesthetized with 0.15 mL 100 mg · mL^{-1} xylazine and 0.05 mL 100mg · mL^{-1} ketamine. They were then given

Table I. Experimental Design for Each Cohort.

COHORT	*N*	8-d PREVENTION OF ACCLIMATIZATION	10-d EXPOSURE	TREATMENT	TREATMENT TYPE
Sea level control	5	Sea level (hypobaric)	Sea level (hypobaric)	Saline	Vector control
High altitude control	5	Sea level (hypobaric)	Altitude (hypobaric)	Saline	Vector control
Established treatment	5	Sea level (hypobaric)	Altitude (hypobaric)	Benzolamide	Carbonic anhydrase inhibitor
Anti-angiogenesis treatment	5	Sea level (hypobaric)	Altitude (hypobaric)	Motesanib	VEGF inhibitor
Pro-angiogenesis treatment	5	Sea level (hypobaric)	Altitude (hypobaric)	Deferoxamine	HIF inducer

30 mg · kg^{-1} Evans Blue (EVBD) via tail vein, which was allowed to circulate for 30 min. During this time interval, the altitude cohorts were placed in hypoxic chambers flushed with 10% oxygen, whereas the sea level cohorts were kept in room air. After the 30-min EVBD circulation time, rats were euthanized with 0.2 mL 26% sodium pentobarbital and craniotomies were performed.

The left cerebral hemispheres were weighed wet, then dehydrated at 65°C and weighed again dry after 1 wk. The right cerebral hemispheres were weighed wet, homogenized in 750 μL of 5% TCA, and after 4 d in room temperature centrifuged for 20 min at 4500 rpm. They were then resuspended in ethanol at 4°C, and after 24 h the centrifugation was repeated. Last, the vortexed right cerebral tissue was assessed with a spectrophotometer at 600 nm against Evan's Blue/formamide stock solutions.

Statistical Analysis

The extravasated Evans Blue pigment, used here as a proxy for cerebral edema, was measured as the absorbance (the unit-less photons absorbed by darkened Evans Blue-laden tissue), adjusted for brain tissue mass (since more mass would incorrectly imply more pigment extravasation if we did not correct for weight).

$$\text{Evans Blue Pigment Absorbance} = \frac{\text{Right Hemisphere Absorbance}}{\text{Right Hemisphere Dry Weight}}$$

where the Right Hemisphere Dry Weight (as this hemisphere was homogenized and used instead for absorbance measurement) was calculated as:

$$\text{Right Hemisphere Dry Weight} = \text{Right Hemisphere Wet Weight} \times \frac{\text{Left Hemisphere Dry Weight}}{\text{Left Hemisphere Wet Weight}}$$

Using the Evans Blue Pigment Absorbance results as our outcome variables, we calculated the group means, SDs, and variance for each group. All results are expressed as means ± 1 SD and verified with histograms to exhibit normal distribution, allowing ANOVA analysis. The ANOVA test[9] was used to compare the variance among means to the variance within each group. The standard *t*-test was used to determine the significance of two-group comparisons of interest. A very small number of *t*-tests were chosen over post hoc tests due to the small number of pairwise comparisons of interest to avoid multiple comparison type 1 error, and to exclude ultimately incidental exploratory pairwise differences. Heterogeneity was assumed significant at $P < 0.05$.

RESULTS

ANOVA results showed group means were significantly heterogeneous ($F_{4,19} = 7.329$, $P = 0.001$). After 10 d of simulated high altitude exposure, we observed significantly less Evans Blue absorbance for the sea level control cohort compared to the high altitude control cohort ($P = 0.019$) (**Fig. 1**). Overall, the high altitude control cohort showed the greatest amount of extravasated Evans Blue absorbance (Fig. 1) when compared to all other groups except the deferoxamine-treated group.

The established treatment cohort, treated with benzolamide while exposed to simulated high altitude, revealed the least Evans Blue absorbance (Fig. 1) among the altitude cohorts, significantly less than the high altitude control cohort ($P = 0.0003$) (Fig. 1). When the established treatment cohort was compared to the sea level control cohort, there was no statistically significant outcome difference ($P = 0.14$).

The anti-angiogenesis-treated cohort, which received motesanib while exposed to simulated high altitude, demonstrated very similar results to the established treatment cohort. There was significantly less extravasated Evans Blue absorbance (Fig. 1) when compared to the high altitude control cohort ($P = 0.029$), and no statistically significant difference compared to the sea level control cohort ($P = 0.39$).

The pro-angiogenesis altitude exposure cohort, treated with the HIF-inducer deferoxamine, exhibited the greatest amount of Evans Blue absorbance (Fig. 1) among all cohorts.

DISCUSSION

The data that show significantly increased extravasation of EVBD from the high altitude control vs. sea level control cohorts demonstrate the protocol was successful in using hypobaric hypoxia to selectively cause cerebrovascular leak in our animal model. That is, the negative control group (sea level control) and the positive control group (high altitude control) validate this study's design, assumptions, and results by exhibiting the expected known outcome at simulated altitude, but not sea level conditions.

The treatment groups showed significant protective effects from the two treatments hypothesized to be beneficial, and exacerbated cerebrovascular leak from the drug hypothesized to worsen hypoxia-induced leak. Benzolamide, a close relative of acetazolamide, was the most effective at preventing cerebrovascular leak—in this case, it normalized the leak back to sea level values. This data corroborates the reasons acetazolamide remains the only FDA-approved prophylaxis for AMS.

Like benzolamide, the anti-angiogenic drug (motesanib) performed as hypothesized by significantly decreasing cerebrovascular leak compared to our high altitude control group. This suggests anti-angiogenic drugs and carbonic anhydrase inhibitors could provide similar HACE prophylaxis, or better prophylaxis in combination—there are several drugs to choose from in each class and these preliminary results show promising outcomes for both. Both were statistically insignificant compared to the sea level control cohort, suggesting benzolamide and motesanib may correct cerebrovascular leak to levels indistinguishable from no altitude exposure at all.

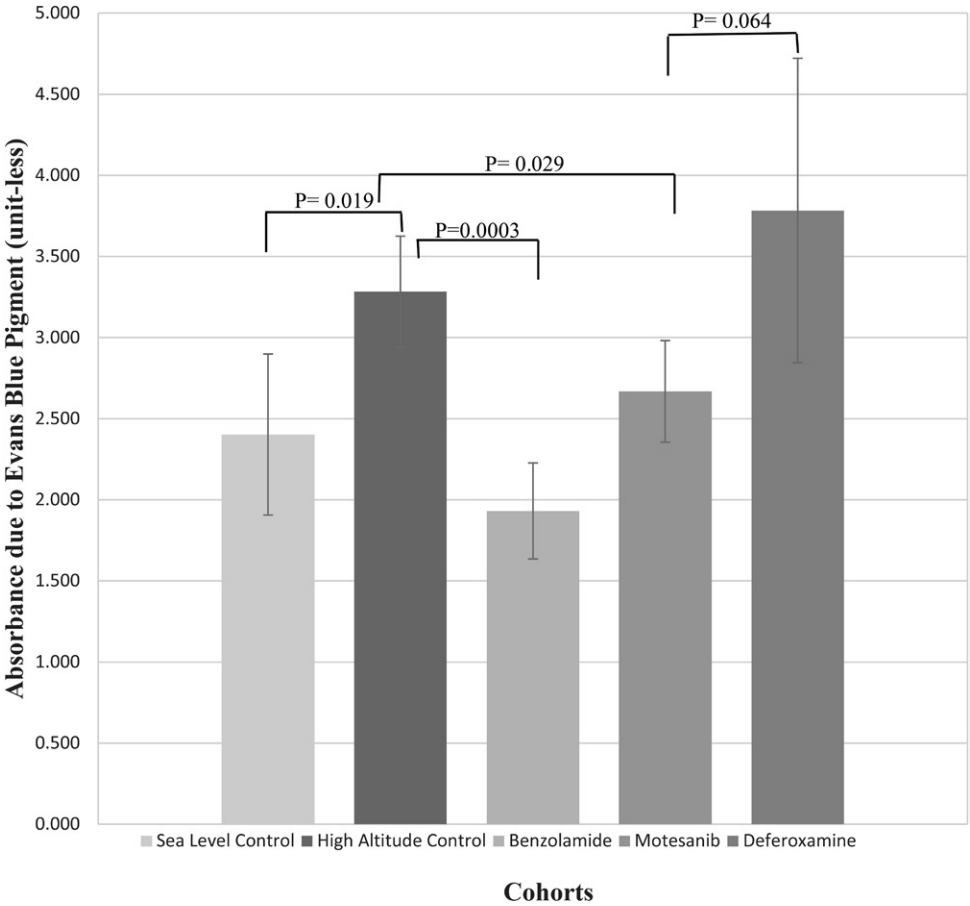

Fig. 1. Quantified cerebrovascular leak for each cohort, measured as spectrophotometer absorbance by extravasated Evans Blue dye in homogenized brain tissue. Each bar represents cohort average ± 1 SD.

In contrast, because HIF-1α is a pro-angiogenesis molecule that promotes VEGF transcription,[10] we expected a HIF-1α promoting drug to increase cerebral leak. As hypothesized, in this study the HIF-inducer, deferoxamine, exacerbated cerebrovascular leak with simulated altitude exposure. These results are congruent with the hypothesized HIF-1α/VEGF angiogenic HACE pathogenesis.

The limitations of this study are primarily: the rat model, limiting our ability to apply findings to humans; the small *N*, limiting our statistical power; and the Evans blue protocol, which is an indirect representation for cerebral edema[6] only if there is actual extravasation of Evans-bound albumin (meaning transudative edema is not noticed) and precise predeath Evans dye blood perfusion (meaning that every anesthetized rat is injected with dye, sacrificed, and harvested for brain tissue within the same amount of heart beats—a goal we strictly maintained to the best of our ability in this study).

Conclusions

This study adopts the less common approach of elucidating disease cause by comparing cohort outcomes resulting from pharmacologically altered disease steps in order to infer pathophysiology from the varying known treatment mechanisms. In this way our experiment gives further evidence in rat models to the angiogenic vascular leak hypothesis of HACE pathogenesis, both by showing the significant protection from cerebrovascular leak in anti-angiogenesis-treated subjects, and the worsened leak among pro-angiogenesis-treated subjects at simulated altitude.

We have also introduced the concept of a novel treatment approach for HACE and AMS prophylaxis using anti-angiogenesis medication as an alternative (or in addition) to carbonic anhydrase inhibitors. Promisingly, anti-angiogenesis medication approved in humans already exists, currently used for diabetic retinopathy, tumor growth delay, and macular degeneration. AMS/HACE are most seen among high altitude athletes, including soldiers and mountaineers,[2] populations already at high risk of potentially deadly dehydration, and with this study we present a new nondiuretic drug class for prophylaxis consideration. Furthermore, adding this already prevalent, nondiuretic pharmaceutical class to the AMS arsenal could provide the first AMS prophylaxis alternative for patients with one of acetazolamide's many contraindications, or decrease the required acetazolamide dose, improving the drug's previously discussed adverse effects and efficacy.

Overall, we have presented statistically significant original research in rats that both provides a new potential mechanism for HACE prophylaxis and submits new evidence for the ongoing HACE pathophysiology debate. Future research in this new direction, with already available anti-angiogenesis medication, may at the least further uncover the root cause(s) of AMS/HACE, and at the most finally relegate HACE tragedies to climbing history.

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