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Safety evaluation of adenosine, lidocaine and magnesium (ALM) intranasal therapy toward human nasal epithelial cells in vitro

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Abstract

Adenosine, lidocaine and Mg^{2+} (ALM) solution is an emerging therapy that reduces secondary injury after intravenous administration in experimental models of traumatic brain injury (TBI). Intranasal delivery of ALM may offer an alternative route for rapid, point-of-care management of TBI. As a preliminary safety screen, we evaluated whether ALM exerts cytotoxic or inflammatory effects on primary human nasal epithelial cells (pHNEC) in vitro. Submerged monolayers and air–liquid interface cultures of pHNEC were exposed to media only, normal saline only, therapeutic ALM or supratherapeutic ALM for 15 or 60 min. Safety was measured through viability, cytotoxicity, apoptosis, cellular and mitochondrial stress, and inflammatory mediator secretion assays. No differences were found in viability or cytotoxicity in cultures exposed to saline or ALM for up to 60 min, with no evidence of apoptosis after exposure to supratherapeutic ALM concentrations. Despite comparable inflammatory cytokine secretion profiles and mitochondrial activity, cellular stress responses were significantly lower in cultures exposed to ALM than saline. In summary, data show ALM therapy has neither adverse toxic nor inflammatory effects on human nasal epithelial cells, setting the stage for in vivo toxicity studies and possible clinical translation of intranasal ALM therapy for TBI treatment.

KEYWORDS
ALM therapy, cytotoxicity, intranasal administration, nasal epithelium, safety, traumatic brain injury

1 | INTRODUCTION

Intranasal administration of neuroprotective drugs for traumatic brain injury (TBI) is an attractive alternative to intravenous delivery since it potentially offers more rapid onset of action by bypassing the blood–brain barrier (BBB).[1,2] Additional advantages of intranasal delivery include: (1) optimal central nervous system (CNS) absorption and bioavailability from a highly vascular nasal epithelium that is innervated by the olfactory and trigeminal nerve pathways; (2) circumvention of first-pass liver degradation and kidney filtration; and (3) a non-invasive

strategy with self-administration potential at the point-of-injury in conscious patients suspected of TBI.[1–3]

Intranasal drug delivery is not without challenges. As the primary site for initial contact, the potential for local adverse effects on the nasal epithelium is an important consideration in drug development.[3] In vitro models using primary human nasal epithelial cell (pHNEC) cultures are widely used to predict biological effects and toxicity potential for intranasal therapies.[1] Adenosine, lidocaine and Mg^{2+} (ALM) is an emerging small-volume drug therapy that has shown neuroprotective effects in experimental models of trauma following intravenous administration.[4–6] In a rat model of moderate TBI, ALM increased survival, reduced release of brain injury markers (neuron specific enolase [NSE], high mobility group box protein-1 [HMGB1]), reduced systemic inflammation, preserved platelet function and corrected coagulopathy.[5] Intranasal delivery of ALM therapy may offer an alternative delivery route for rapid, point-of-care management of TBI. Importantly, A, L or M individually do not confer the multi-protection properties of combined ALM.[7–9] As an initial safety assessment, the aim of this study was to examine the effect of ALM on the viability, cytotoxicity and inflammatory responses of pHNEC in submerged and air–liquid interface (ALI) cultures. We hypothesized that cellular responses would be comparable following short-term exposure of submerged and ALI pHNEC cultures to ALM and its excipient, normal saline (0.9%).

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Nasal epithelial cells were obtained from two healthy donors on four separate occasions (Donor 1, single collection; Donor 2, collection on three separate occasions) following informed consent. The study protocol was approved by the Institutional Human Research Ethics Committee (H8967) and followed the ethical guidelines of the Code of Ethics (Declaration of Helsinki) of the World Medical Association for trials involving humans. The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[10]

2.2 | Primary human nasal cell (pHNEC) collection and submerged, monolayer culture conditions

Nasal epithelial brushings were taken from the middle meatus of the left and right nostrils using a sterile cytology brush (McFarlane Medical) and immediately suspended in phosphate-buffered saline (PBS). Following centrifugation (200*g*, 5 min), cells were resuspended with 5 ml of PneumaCult-Ex Plus media (PCEM, STEMCELL Technologies) supplemented with foetal bovine serum (10% FBS, Sigma-Aldrich) and antibiotic/antimycotic solution (1%, Thermo Fisher Scientific), seeded directly onto placental collagen-coated (0.8 μg/cm^2; C5533, Sigma-Aldrich) T25 culture flasks (Thermo Fisher Scientific), and cultured for 24 h at 37°C in 5% CO_2. Non-adherent cells were removed after 24 h of culture, with further media changes (FBS-free) performed three times weekly until cultures reached 80% confluence (~Day 8). pHNEC were enzymatically lifted (0.25% trypsin–EDTA; Thermo Fisher Scientific), washed and subcultured in collagen-coated T75 culture flasks. Secondary cultures reached 80%–90% confluence within 7 days. For assessment of cellular responses in submerged cultures, pHNEC were enzymatically lifted from T75 flasks, washed and seeded into 96-well plates at 10^4 cells/well. Following overnight incubation to allow cell attachment, cells were washed once prior to commencing treatment exposure experiments, as described below.

2.3 | Air–liquid interface (ALI) cultures of pHNEC

To establish ALI cultures, pHNEC were seeded at a density of 6×10^4 cells per 12 mm polyester transwell insert (0.4 μm pore size; Corning, NY) in PneumaCult-ALI medium and following the ALI culture standard protocol from STEMCELL Technologies. Cultures were maintained in the presence of 5% CO_2 at 37°C, with apical and basal chamber media replaced every second day until cells reached 100% confluence (~Day 8). To commence ALI culture, media was removed from the apical chamber and replaced in the basal chamber every second day. Cells in the apical chamber were washed once per week with pre-warmed sterile Hanks' Balanced Salt Solution (HBSS), and all liquid was aspirated. ALI differentiation of pHNEC occurred ~24 days after airlifting, with cultures subsequently used for assessment of cellular responses to treatment exposure as described below.

2.4 | Treatments

Adenosine (A9251), Lidocaine-HCl (L5647), and $MgSO_4$ (M7506) were purchased from Sigma-Aldrich. Cultures were exposed to (1) media only (control), (2) saline only (0.9% NaCl), (3) a therapeutic ALM solution (adenosine

1 mM, lidocaine 3 mM, Mg^{2+} 2.5 mM) or (4) a supratherapeutic, double-strength (DS) ALM solution (adenosine 2 mM, lidocaine 6 mM, Mg^{2+} 5 mM). Submerged monolayers were exposed to treatments in triplicate and ALI pHNEC cultures in duplicate for 15 or 60 min (37°C, 5% CO_2). For ALI pHNEC experiments, responses to DS ALM exposure were assessed after a contact time of 15 min. Treatments (0.5 ml) were applied to the apical chamber of ALI cultures. Exposure times were selected to reflect and exceed the maximum expected time of direct exposure of cells to therapeutic concentrations of ALM after intranasal delivery. Since the average nasal mucociliary clearance transit time in humans is ~15 min, this exposure period was considered representative of the contact time for therapeutic ALM within the human respiratory mucosa.[11] After treatment exposure, supernatants were removed and stored at −80°C for subsequent cytokine analysis. Cells were washed, media replenished, and submerged monolayer and ALI pHNEC cultures were incubated for a further 24 h prior to assessment of viability, cytotoxicity and inflammatory responses.

2.5 | Cell viability

The cell counting kit-8 (CCK-8) kit (Sigma-Aldrich) was used to measure cell viability 24 h after treatment exposure, according to the manufacturer's protocol. Staurosporine (STS), an inducer of apoptosis, was included as a positive control for all experiments (10 μM, 4 h). WST-8 (10% v/v) was added to each well 24 h after treatment exposure, and cultures were incubated for 2 h at 37°C. For ALI-pHNEC cultures, WST-8 was added to the basal chamber with media transferred to a 96-well plate in triplicate for viability assessment. Absorbance was measured at 460 nm using a POLARstar Omega microplate reader (BMG Labtech). Relative viability was calculated as the absorbance ratio of treated and control cells, with data expressed as a percentage.

2.6 | Cytotoxicity

Cytotoxicity was measured using a lactate dehydrogenase (LDH) release assay, according to manufacturer protocols (CyQUANT LDH Cytotoxicity Assay Kit, Invitrogen). Relative cytotoxicity was calculated based on LDH levels of negative controls (untreated cells) and maximum release controls (untreated and lysed cells), with data expressed as a percentage.

2.7 | Mitochondrial membrane potential (MMP)

To quantify changes in MMP in submerged pHNEC monolayer cultures after treatment exposure, a TMRE-Mitochondrial Membrane Potential Assay Kit (Abcam) was used according to manufacturer protocols. Tetramethylrhodamine ethyl ester (TMRE) is a cell-permeant, positively charged, red-orange dye that readily accumulates in active mitochondria due to its relative negative charge. As a positive control, we included carbonyl cyanide-p trifluoromethoxy phenylhydrazone (FCCP; 20 μM, 4 h incubation), an ionophore uncoupler of oxidative phosphorylation that reduces MMP and TMRE staining. Adherent pHNEC cultures in black 96-well plates were stained with TMRE (100 nM) for 20 min, then washed in HBSS prior to the addition of treatments. HBSS was added to the unstimulated control wells. After 15 min, fluorescence was measured at Ex/Em 549/575 nm using a POLARstar Omega microplate reader.

2.8 | Inflammatory cytokines

Human cytometric bead array (CBA) Standard and Enhanced Sensitivity Flex Sets (BD Biosciences) were used to measure concentrations of interleukin (IL)-1α, IL-1β, tumour necrosis factor (TNF)-α, IL-6, IL-8 and monocyte chemoattractant protein (MCP)-1 in cell-free supernatants from control and treated wells. Assays were carried out according to the manufacturer's instructions with samples acquired on a FACSCanto II flow cytometer (BD Biosciences) and FlowJo v.10.3 software used for data analysis.

2.9 | Apoptosis

Caspase-3/7 activity was measured in submerged pHNEC after treatment exposure using the Caspase-Glo® 3/7 assay (Promega) and according to the manufacturer's protocol. Briefly, treatments were added to adherent, submerged pHNEC in white 96-well plates (10^4 cells/well) for 15 or 60 min. STS (10 μM) was added to the control wells for 4 h. Following exposure, cells were washed, media replaced and Caspase-Glo® 3/7 reagents added. Plates were incubated at RT for 1 h with shaking (300 rpm), then luminescence measured using a POLARstar Omega microplate reader.

2.10 | Cellular oxidative stress

To estimate oxidative stress and production of cellular reactive oxygen species (ROS) within submerged pHNEC after treatment exposure, we used a DCFDA/H2CFDA Cellular ROS Assay Kit (Abcam) and measured fluorescence by microplate spectrophotometry according to manufacturer protocols. The cell-permeant reagent, 2′,7′-dichlorofluorescin diacetate (DCFDA), freely enters the cell, is hydrolysed by intracellular esterase into its non-fluorescent form, and then, in the presence of intracellular ROS, becomes oxidized into highly fluorescent 2′,7′-dichlorofluorescein. Positive control wells were stimulated with ter-butyl hydrogen peroxide (TBHP, 100 μM), an ROS generator, for 4 h. Briefly, adherent pHNEC in black 96-well plates (10^4 cells/well) were stained with DCFDA (10 μM) for 30 min, washed in HBSS and treatments added. Negative control wells contained HBSS only. Fluorescence was measured 15 and 60 min after the addition of treatments to cultures using a POLARstar Omega microplate reader at Ex/Em 485/535 nm.

2.11 | Histochemistry and histology

To enable visualization of submerged pHNEC following treatment exposure, cells were cultured in 8-well culture slides. Twenty-four hours after treatment exposure, submerged pHNEC were fixed with 1% paraformaldehyde (PFA) for 1 h at room temperature (RT), washed in HBSS and then stained following the May-Grünwald–Giemsa procedure. To enable visualization of ALI-pHNEC cultures following treatment exposure, cultures were fixed with 4% PFA for 24 h at 4°C. Fixed membranes were excised from insert cups, processed and paraffin embedded for histology according to the protocol of Manna & Cardonna (STAR Protocols 2 2021). Sections (5 μm) were cut, stained with haematoxylin and eosin (H&E) and visualized by light microscopy (Nikon Eclipse i50).

2.12 | Statistics

GraphPad Prism 10.1.1 was used for all data analysis and graphing. Normality assumptions and equality of variances were assessed using Shapiro–Wilk and Levene's tests, respectively. Between-group responses were compared using analysis of variance (ANOVA), with a Tukey honestly significant difference (HSD) post hoc test. Data are presented as the mean ± standard error of the mean (SEM) of at least three independent experiments, with significance set at $p < 0.05$.

3 | RESULTS

3.1 | Effects of ALM exposure on cell viability and cytotoxicity in submerged pHNEC monolayers

Compared to normal saline (0.9% NaCl), viability was equivalent following exposure of submerged pHNEC to therapeutic ALM for up to 60 min (Figure 1A). There was no adverse effect on cell viability following exposure of pHNEC to DS ALM for 15 min, with viability decreasing by ~30% when exposure times were extended to 60 min. Similarly, no significant cytotoxic responses were observed for ALM or DS ALM, even with prolonged exposure times (Figure 1B), with comparable cellular morphology between treatment groups in submerged pHNEC cultures (Figure 1C).

3.2 | Effects of ALM exposure on cellular stress and inflammatory responses in submerged pHNEC monolayers

Caspase-3/7 activity (Figure 2A) and MMP (Figure 2B) were comparable between control (untreated) pHNEC cultures and those exposed to ALM or normal saline for 15 min. Cellular ROS levels, however, were significantly lower in pHNEC cultures exposed to ALM for 15 min compared to those exposed to saline ($p = 0.01$; Figure 2C). Assessment of key inflammatory mediators in pHNEC culture supernatants immediately following 15-min treatment exposure found no significant differences in IL-1α, IL-1β, IL-6, TNF-α, IL-8 or MCP-1 concentrations between untreated control cultures or those exposed to ALM or DS ALM (Figure 2D–I). Similarly, 24 h concentrations of inflammatory mediators remained equivalent in untreated pHNEC cultures and those exposed to therapeutic or supratherapeutic ALM for either 15 (Figure 3A–F) or 60 min (Figure 3G–L).

3.3 | Effects of ALM exposure on cell viability, cytotoxicity and inflammatory responses in ALI-pHNEC cultures

To model the differentiated, pseudostratified mucociliary structure of the human nasal epithelium, ALI pHNEC was generated using collagen-coated transwell inserts and differentiation over a 24-day period (Figure 4A). Histologically, there was no evidence of cellular damage or loss of structural integrity of the epithelium following 15 min of exposure to saline, ALM or DS ALM (Figure 4B). Compared to normal saline, relative viability

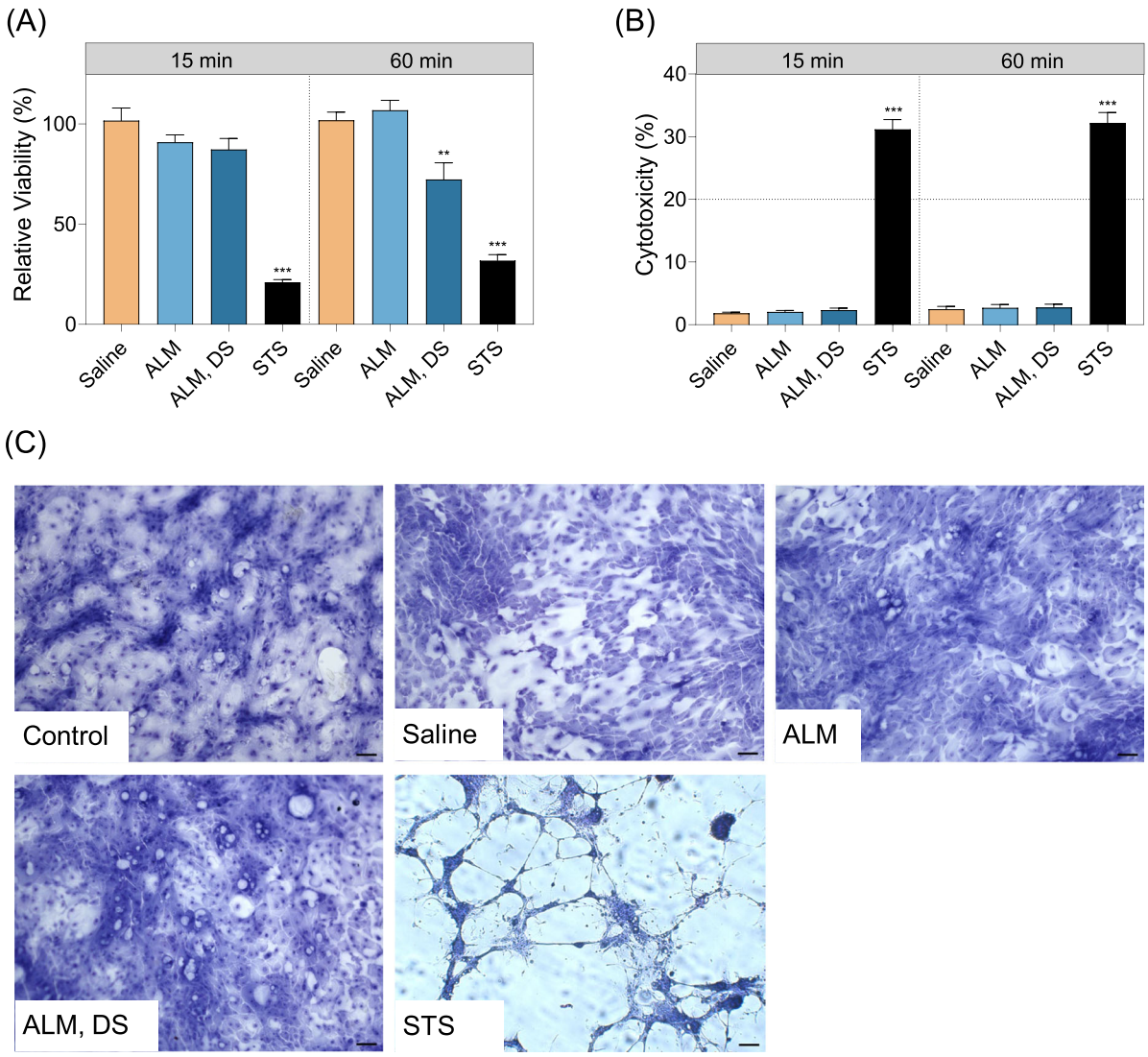

FIGURE 1 Viability and cytotoxicity in submerged cultures. (A) Relative viability, and (B) cytotoxic response of submerged primary human nasal epithelial cell (pHNEC) cultures 24 h after exposure to media only (control), saline only, adenosine, lidocaine and Mg^{2+} (ALM) (therapeutic), double-strength (DS) ALM (supratherapeutic) for 15 or 60 min. Positive control cultures were stimulated with staurosporine (STS) for 4 h. Data is expressed as mean ± standard error of the mean (SEM). $^{**}p < 0.001$ compared to control. (C) Representative images of May-Grünwald–Giemsa-stained pHNEC cultures 24 h after exposure to media only (control), saline only, ALM (therapeutic), or DS ALM (supratherapeutic) for 15 min, or STS for 4 h. Scale bar indicates 100 μm.

was equivalent following exposure of ALI-pHNEC cultures to therapeutic ALM for 15 or 60 min (Figure 4C). Viability was significantly greater in cultures exposed to supratherapeutic DS ALM than saline ($p = 0.005$) and therapeutic ALM ($p = 0.006$) for 15 min. For saline-exposed cultures, viability was improved following exposure times of 60 min, compared to 15 min ($p = 0.002$). Cytotoxicity levels were comparable between control and treatment groups 24 h after exposure, with no adverse effect after 60 min (Figure 4D). Levels of secreted inflammatory mediators in ALI-pHNEC cultures 24 h after exposure to ALM or DS ALM for 15 min were equivalent to those in untreated control cultures (Figure 4E–J).

4 | DISCUSSION

Intranasal administration of ALM offers a potential rapid, non-invasive delivery mode to provide neuroprotection after TBI. We report for the first time that ALM has no adverse effects on the morphology or functional responses of pHNECs following contact times of up to 1 h. A direct cell contact time of 1 h far exceeds the tissue contact times associated with intranasal delivery of ALM.

The three individual actives of ALM therapy (adenosine, lidocaine and magnesium) are FDA-approved for their specific indications and have each been safely

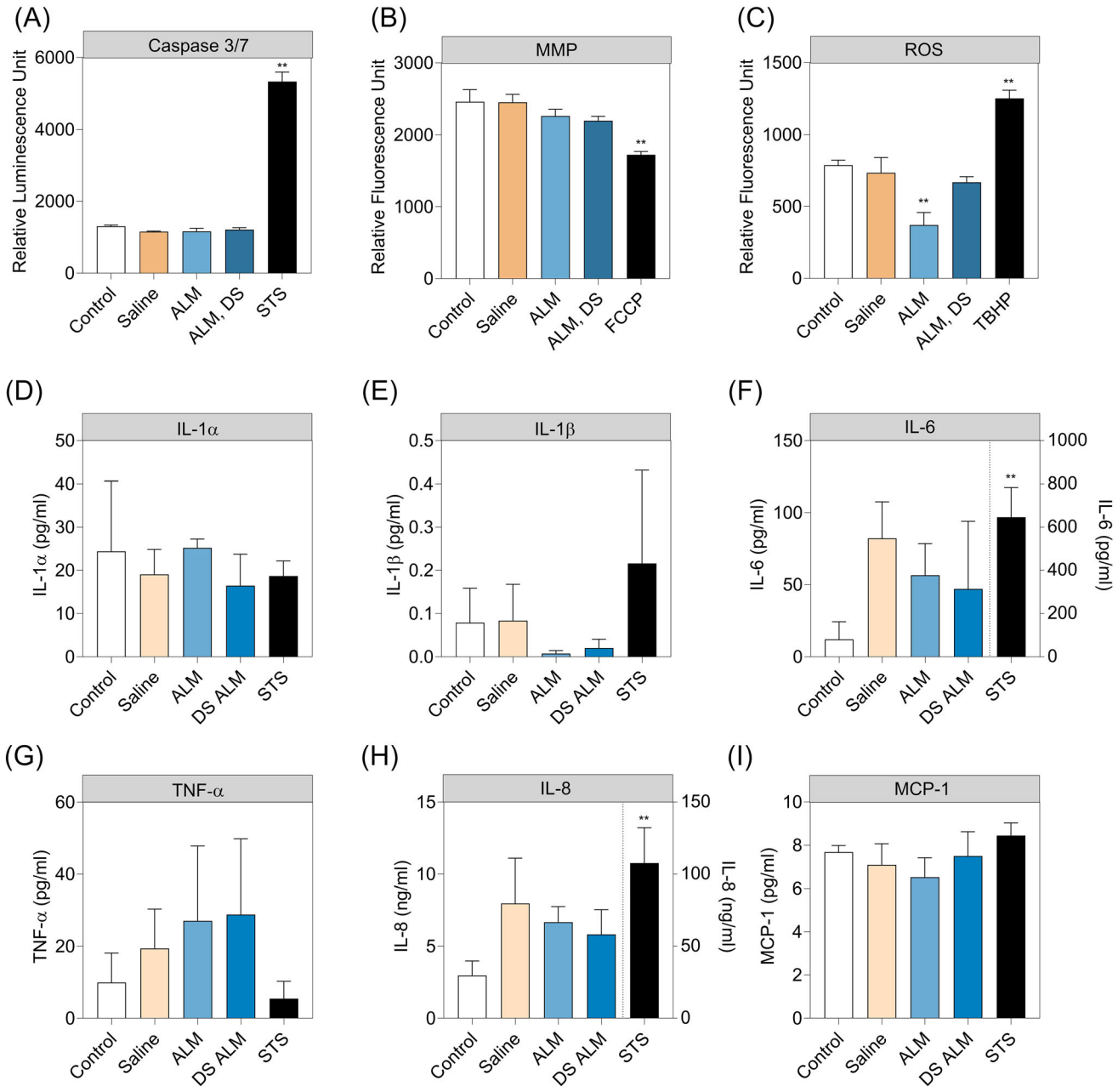

FIGURE 2 Cellular activation and stress responses in submerged cultures. (A) Caspase-3/7 activity, (B) mitochondrial membrane potential (MMP), (C) cellular reactive oxygen species (ROS) production, and concentration of (D) interleukin (IL)-1α, (E) IL-1β, (F) IL-6, (G) tumour necrosis factor (TNF)-α, (H) IL-8 and (I) monocyte chemoattractant protein (MCP)-1 in culture supernatants immediately following 15 min exposure to media only (control), saline only, adenosine, lidocaine and Mg^{2+} (ALM) (therapeutic), or DS ALM (supratherapeutic). Positive control cultures were stimulated with staurosporine (STS) for 4 h. Data is expressed as mean ± standard error of the mean (SEM). $^{**}p < 0.01$ compared to control.

administered via intravenous, intra-articular and intra-osseous routes.[12–24] Intranasal administration of 2%–10% lidocaine is also often used for pain management associated with migraines and seizures in both adults and children.[25,26] The ALM combination, however, has not been tested for intranasal delivery. A key safety consideration for clinical translation of intranasal ALM therapy is the absence of potential toxicity to the nasal epithelium.

In the present study, both submerged and ALI cultures of pHNEC showed a similar pattern of functional responses to ALM. In contrast to immortal cell lines, primary human epithelial cell cultures are preferred for in vitro toxicity screening as they more closely reflect the heterogenous cell types within the nasal epithelium.[27–29] Under ALI culture conditions, pHNEC differentiate into an epithelial layer comprising ciliated pseudostratified

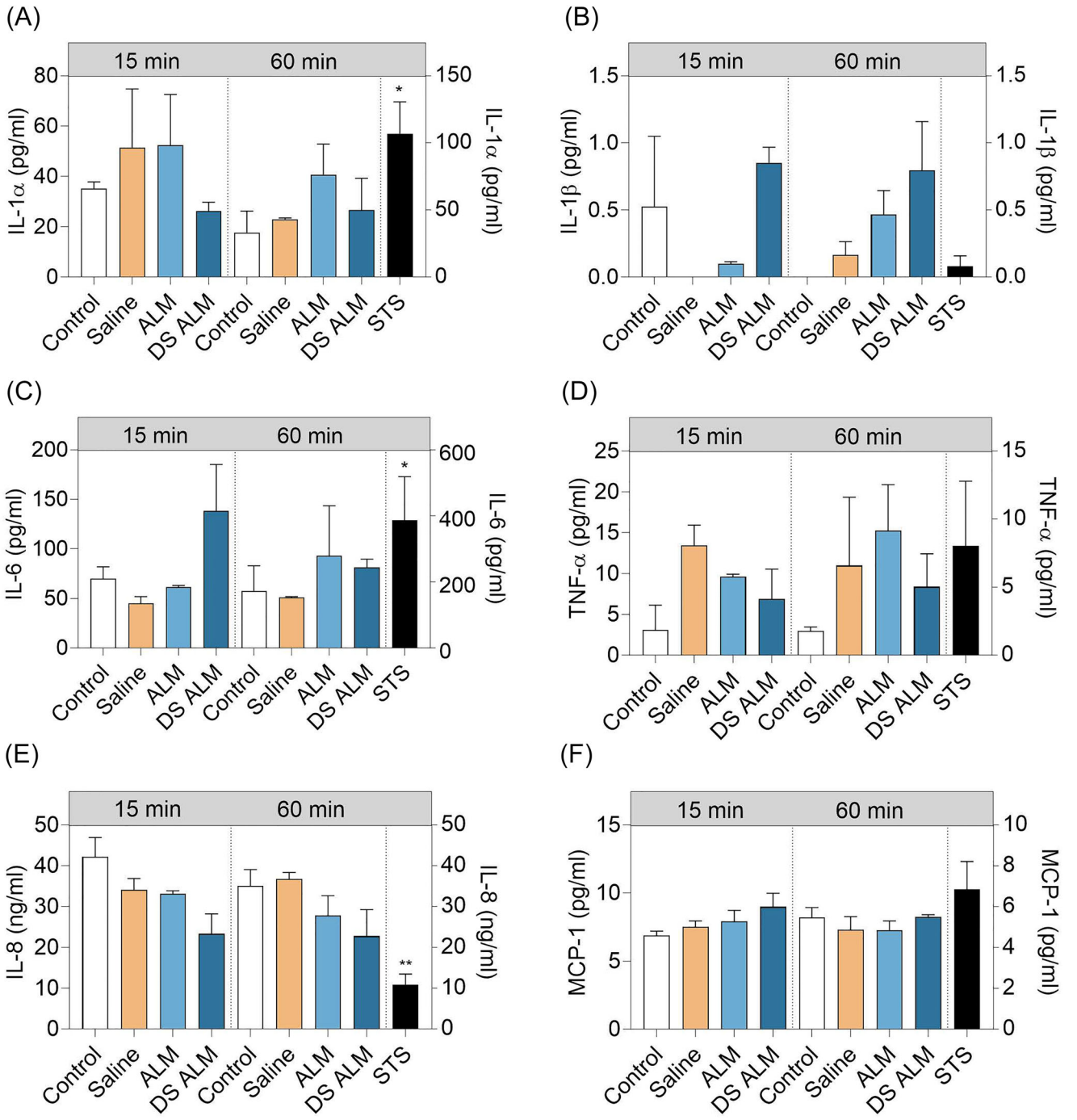

FIGURE 3 Secreted inflammatory mediator profiles in submerged cultures. Concentration of (A) interleukin (IL)-1α, (B) IL-1β, (C) IL-6, (D) tumour necrosis factor (TNF)-α, (E) IL-8 and (F) monocyte chemoattractant protein (MCP)-1 in culture supernatants 24 h after exposure to media only (control), saline only, adenosine, lidocaine and Mg^{2+} (ALM) (therapeutic), or DS ALM (supratherapeutic) for 15 or 60 min. Positive control cultures were stimulated with staurosporine (STS) for 4 h. Data is expressed as mean ± standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$, compared to media only (control).

columnar cells, non-ciliated mucous-producing goblet cells and basal cells that exhibits structural, physiologic, metabolic and inflammatory features closely resembling the human nasal mucosa.[27–29] As the major interface between the environment and host, nasal epithelial cells mount a potent, protective innate immune response to foreign stimuli, including the release of inflammatory mediators such as ROS, leukocyte-attracting chemokines (IL-8, MCP-1) and proinflammatory cytokines (IL-1α, IL-1β, IL-6 and TNF-α).[27,30] A key finding of the present study was the absence of an inflammatory stress response following in vitro exposure of pHNEC cultures to ALM

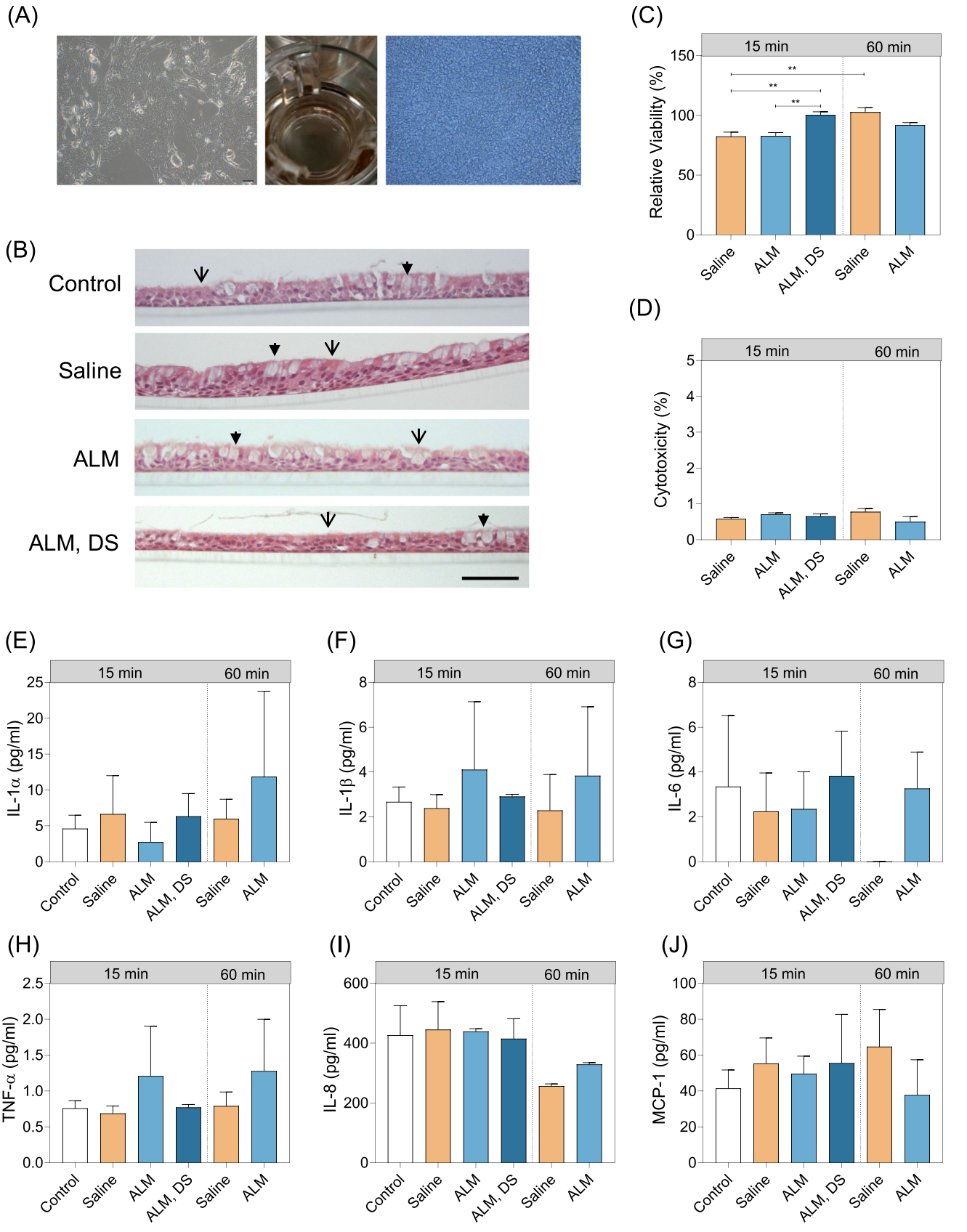

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FIGURE 4 Cell viability, cytotoxicity and inflammatory responses of air–liquid interface (ALI) cultures. (A) ALI cultures of primary human nasal epithelial cell (pHNEC) were generated using collagen-coated transwell inserts, with differentiation occurring over a 24-day period. (B) The resulting multilayer formation closely resembled native tissue architecture (cilia, arrows; goblet cells, arrowheads), with no histological evidence of structural integrity loss, 24 h after exposure to media only (control), saline only, adenosine, lidocaine and Mg^{2+} (ALM) (therapeutic), or DS ALM (supratherapeutic) for 15 min. Scale bar indicates 100 μm. (C) Relative viability and (D) cytotoxic responses of cultures exposed to saline, ALM or DS ALM for 15 or 60 min. Concentrations of (E) interleukin (IL)-1α, (F) IL-1β, (G) IL-6, (H) tumour necrosis factor (TNF)-α, (I) IL-8 and (J) monocyte chemoattractant protein (MCP)-1 in culture supernatants 24 h after exposure to media only (control), saline only, ALM (therapeutic) or DS ALM (supratherapeutic) for 15 or 60 min. Data is expressed as mean ± standard error of the mean (SEM). $^{**}p < 0.01$.

for up to 1 h, with cytokine and chemokine concentrations comparable to those exposed to normal saline. In fact, ALM exposure significantly lowered cellular ROS levels in pHNEC compared to normal saline. This finding is consistent with previous reports of antioxidant activity in multiple cell types following exposure to the individual constituents, adenosine,[31] lidocaine[32] and magnesium,[33] as well as reduced pulmonary oxidative stress in ALM-treated animals in a rat model of severe thermal injury.[34] Further studies are needed to explore this antioxidant effect and how it contributes to ALM's multi-pronged protection against secondary injury progression following sterile and non-sterile injury in vivo.[8,35]

In line with the absence of inflammatory and oxidative stress, we also found no evidence of mito- or cytotoxicity after exposure of pHNEC to therapeutic or supratherapeutic concentrations of ALM. Drug-induced oxidative stress is a common mechanism of toxicity in numerous tissues, with ROS damaging critical cellular components at the molecular level and negatively impacting cell survival.[36] MMP drives the synthesis of ATP and is a key indicator of mitochondrial function, with decreases in MMP linked to activation of caspase-mediated apoptotic pathways.[37,38] In the present study, MMP and caspase-3/7 activity within pHNEC cultures remained unchanged after ALM exposure, suggesting that even at supratherapeutic concentrations, ALM does not induce mitochondrial dysfunction or apoptosis. Similarly, LDH levels remained below 3% for ALM-treated pHNEC cultures and were comparable to those exposed to saline. Typically, LDH-based cytotoxicity is determined at a cut-off of 20%.[39] Findings were supported histologically, with no evidence of cellular necrosis or loss of structural integrity in ALI cultures.

A potential limitation of our study is the use of isolated human respiratory cells in ex vivo cultures, which may not reflect the complexities of the same cells within the highly vascularized and innervated environment of the nasal and olfactory epithelium. Preclinical safety studies, including histological assessments of brain and local respiratory and olfactory tissue, are currently in progress. A second limitation is the inclusion of two donors for the generation of pHNEC cultures, albeit with collections performed on multiple, separate occasions. Genetic diversity in future in vivo safety assessments of ALM will be an important consideration for clinical translation. Notably, the absence of any deleterious effects of ALM toward human nasal epithelial cells in the present study is consistent with findings using human platelets[40] and human mesenchymal stem cells.[41]

Despite decades of research, no drug has been shown to significantly improve patient outcomes after TBI. The key to successful therapy is the early attenuation of neuroinflammation triggered by TBI to prevent secondary injury progression in the brain and other organs. ALM is a systems-acting therapy that appears to confer whole-body protection after TBI, as well as haemorrhage, thermal injuries, major surgery and infection.[8,42] ALMs protective properties within different trauma and infective states involve shifting CNS sympathetic hyperactivity to parasympathetic dominance, reducing inflammation, improving tissue O_2 supply and preserving mitochondrial function.[4,8,35,42] Intranasal administration of ALM therefore offers a promising strategy to rapidly blunt damaging inflammatory and immune responses triggered by TBI. Importantly, ALM's protection is not conferred using A, L or M alone; it is only the combination of actives in vivo that appears to switch the "injury" phenotype to a "protective" phenotype. For this reason, elucidation of the molecular basis for ALM's protective switch within different pathophysiological states, including after TBI, is reliant on further in vivo investigations, which is the focus of future research.

We conclude that ALM does not exert adverse cytotoxic, cellular stress or inflammatory effects on human nasal epithelial cells in vitro. This data provides support for the transition to human safety trials, with potential for intranasal delivery of ALM therapy for early treatment of TBI in the conscious patient.

AUTHOR CONTRIBUTIONS

Jodie L. Morris, Hayley L. Letson and Geoffrey P. Dobson contributed to the conception and design of the study. Experiments and data analyses were performed by Jodie L. Morris. All authors contributed to the interpretation of the data, manuscript preparation and review.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no competing interests.

DATA AVAILABILITY STATEMENT

Datasets are available from the corresponding author upon reasonable request.

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