

AP39, a novel mitochondria-targeted hydrogen sulfide donor, promotes cutaneous wound healing in an *in vivo* murine model of acute frostbite injury

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ABSTRACT

Frostbite injury refers to cold tissue injury which typically affects the peripheral areas of the body, and is associated with limb loss and high rates of morbidity. Historically, treatment options have been limited to supportive care, leading to suboptimal outcomes for affected patients. The pathophysiology of frostbite injury has been understood in recent years to share similarity with that of cold ischemia-reperfusion injury as seen in solid organ transplantation, of which mitochondria play an important contributing role. The present study investigated whether AP39, a novel mitochondria-targeted slow-releasing hydrogen sulfide donor, applied topically in a vehicle cream at 200 nM or 1 μM could mitigate frostbite injury and promote wound healing in mice. Frostbite injury was induced continuously for 3 min on the dorsal skin of C57BL/6 mice (*Mus musculus*) using magnets frozen on dry ice (-80 °C). AP39, delivered via a vehicle cream, was used daily to treat frostbite injury until animals were euthanized on day 15 after induction of frostbite injury. Wound tissues were stained with hematoxylin and eosin along with immunofluorescence staining with cleaved caspase-3, CD31, KI-67, CD163, fibronectin and cytokeratin. While 200 nM AP39 improved granulation tissue maturation ($p < 0.001$), angiogenesis ($p < 0.01$) and cell proliferation ($p < 0.001$) compared to vehicle control, 1 μM AP39 further increased granulation tissue formation compared to other frostbite groups ($p < 0.001$). Thus, AP39 promoted frostbite wound healing, and therefore could be considered as a treatment option for patients with frostbite injury.

1. Introduction

Frostbite is a debilitating hypothermic injury that occurs when tissue fluids crystallize as a result of exposure to temperatures below their freezing point [1], causing damage and protein changes in those tissues [2]. As tissues freeze faster at lower temperatures, the severity of

frostbite injury is proportional to duration of exposure to low temperatures. Frostbite injury has historically affected primarily military personnel. The epidemiology of frostbite has changed over the last 20 years and it is now known to affect the civilian population at a high frequency [1]. The increasingly common incidence of frostbite in the general population is raised by homelessness, occupational accidents,

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recreational incidents and inadequate home heating [3]. Although frostbite injury is not typically associated with high rates of mortality, high rates of morbidity are often associated with cases of severe frostbite, which can result in limb loss. Frostbite injury primarily affects extremities of the body, with 90 % of injuries affecting hands and feet [3], and has social and economic consequences for affected individuals, particularly in cases of limb loss.

Frostbite injury shares pathophysiological similarity with cold IRI as has always been encountered in solid organ transplantation. In addition, there are studies which also described direct cellular damage caused by ice crystal formation as well as progressive dermal ischemia, which is mediated by release of norepinephrine from cold-sensitive afferent neurons, resulting in localized vasoconstriction and subsequent ischemia [4–6]. Frostbite injury occurs in four phases, the first of which is known as the pre-freeze phase involving both vasoconstriction and dermal ischemia [6]. In humans, skin displays a response to temperatures below 15 °C called the “adaptive hunting reaction” [5]. This reaction occurs in the pre-freeze phase and allows for initial vasoconstriction followed by cycling of vasodilation, regulated by smooth muscle, in an attempt to protect vital organs through maintenance of core temperature [7]. Next is the freeze-thaw phase when freezing of tissue begins to occur and extracellular ice crystals are initially formed. Subsequently, intracellular ice crystals begin to form as the tissue temperature continues to fall. During these processes, the cell membrane becomes damaged and the osmotic gradient is disrupted, which leads to disruption of the electron transport chain in mitochondria [8]. As ice crystals expand intracellularly, the cells swell and eventually die via mechanical destruction. Partial thawing in this phase may initiate ischemia-reperfusion injury (IRI). During reperfusion, return of blood to the tissue following the cold ischemic period results in inflammation and further tissue damage through induction of oxidative stress due to overproduction of reactive oxygen species (ROS) from mitochondria [5,9]. Additionally, immune cells are activated by ligands on necrotic and apoptotic cells, which mediate an amplified inflammatory response, leading to endothelial damage [10]. The next phase is the vascular stasis phase during which there may be continued fluctuation between dilation and constriction of vessels, as well as increased permeability of the vessels. During this phase, blood viscosity increases due to increased amounts of pro-coagulating factors that are released by the endothelial cells in the vascular wall in response to damage [11]. The final phase is the late ischemia phase during which tissue necrosis occurs following thrombus-induced inflammation [6]. At the subcellular level, frostbite decreases adenosine triphosphate (ATP) production and depletes energy reserves, reduces 6-phosphogluconate dehydrogenase activities as well as mitochondrial and soluble hexokinase content in skin [37,38].

There is currently no effective way to treat frostbite injury apart from supportive care such as rewarming of tissue, daily wound care and treatment with analgesics [12]. Therefore, there is urgent need to identify novel pharmacological agents for effective treatment. Hydrogen sulfide (H₂S) has recently been established to possess therapeutic properties when delivered at low physiological concentrations using donor compounds in various animal models of human diseases [13]. We and others previously reported protection against cold IRI by the H₂S donor compound, AP39, in murine and porcine models of kidney and heart transplantation [14–17]. Given the overwhelming mitochondrial component in IRI in organ transplantation, and IRI involvement in frostbite injury, we postulated that mitochondrial protection with H₂S (via AP39) could also be protective in frostbite injury. Therefore, in this study, we aimed to extrapolate the beneficial impact of H₂S therapy in IRI of transplantation to a validated model of frostbite injury and investigate whether AP39 therapy could promote wound healing in mice exposed to dorsal skin frostbite.

2. Materials and methods

2.1. Ethical statement and animal grouping

The Animal Use Committee of the University of Western Ontario approved all experimental procedures. The experimental procedures were considered as Category D from the Canadian Council of Animal Care’s Categories of Invasiveness for Animal Experiments. A total of 25 B57BL/6 mice aged between 8 and 10 weeks old were obtained from Charles River Canada (Senneville, Quebec, Canada) and housed 5 per cage under a 12-hour light/dark cycle at the Health Sciences Animal Care Facility at the University of Western Ontario (London, Ontario, Canada). Mice were randomly assigned to 5 groups being healthy control (n = 5), crush control (n = 5), vehicle control (n = 5), 200 nM AP39 (n = 5) and 1 µM AP39 (n = 5).

2.2. Skin preparation

To ease the process of skin preparation, mice were initially anesthetized with a combination of 20 mg/mL xylazine and 100 mg/mL ketamine intraperitoneally, and the dorsal fur of the animals were shaved using electric clippers from the top of the hind legs to the base of the neck. A depilatory cream was then used to remove any fur still remaining. General anesthesia was maintained by inhalation of 5 % isoflurane and oxygen delivered at 400 mL/min to minimize the time of recovery post-anesthesia. Following complete fur removal, the skin of the animal was cleaned completely of the hair removal cream using an alcohol prep pad. Next, a silicone sheet was used as a template to trace a uniform circle on the surface of the skin using a permanent marker. The circular outline on the back of the animal was used as a guide during frostbite injury (Fig. 1).

2.3. Cream preparation and application

A skin cream compound was used as a vehicle to deliver AP39 in order to penetrate the stratum corneum (outermost layer of the skin), which acts as a physical barrier. The vehicle cream, containing hyaluronan and phosphatidylethanolamine (a phospholipid) as active ingredients, was provided by Dr. Eva Turley, and allowed for delivery of AP39 to the vascularized layers of the dermis [18]. AP39 was synthesized as previously described [19], and diluted to a stock concentration of 1 mM in DMSO. To prepare the cream+AP39 compound, the AP39 was added via micropipette combined by stirring and subsequent mixing using a vortex device to final concentrations of 200 nM and 1 µM.

2.4. Induction of frostbite injury

The protocol of the frostbite model is illustrated in Fig. 1A. Based on a previously validated model [2], 100 % ceramic magnets (diameter 18.0 mm, thickness 5.0 mm, weight 4.5 g) were used to induce frostbite injury. Magnets were frozen in dry ice (-80 °C) prior to animal skin preparation. The dorsal skin of the mouse was pinched to create a skin fold and a silicone sheet was placed underneath as illustrated in Fig. 1B. The silicone sheet acted as a barrier to prevent the mice from possible systemic hypothermia while creating a precise injury and maximizing survival. Two frozen magnets were then placed on the skin from opposite sides of the skin fold such that they adhered with the middle of the circle outline at the peak of the fold’s center as described by Auerbach et al. [2] and illustrated in Fig. 1B. After 1 min, the magnets were removed and new frozen magnets simultaneously placed in order to keep the freezing temperature of the magnets consistent and ensured that no thawing of the skin occurred. A total of 3 magnet exchange cycles occurred in order to induce frostbite injury to the mice for 3 min after which the skin was allowed to thaw completely. Core temperature of each mouse was continuously monitored using a rectal thermometer and did not change. The animals were placed on a heating pad set to 37

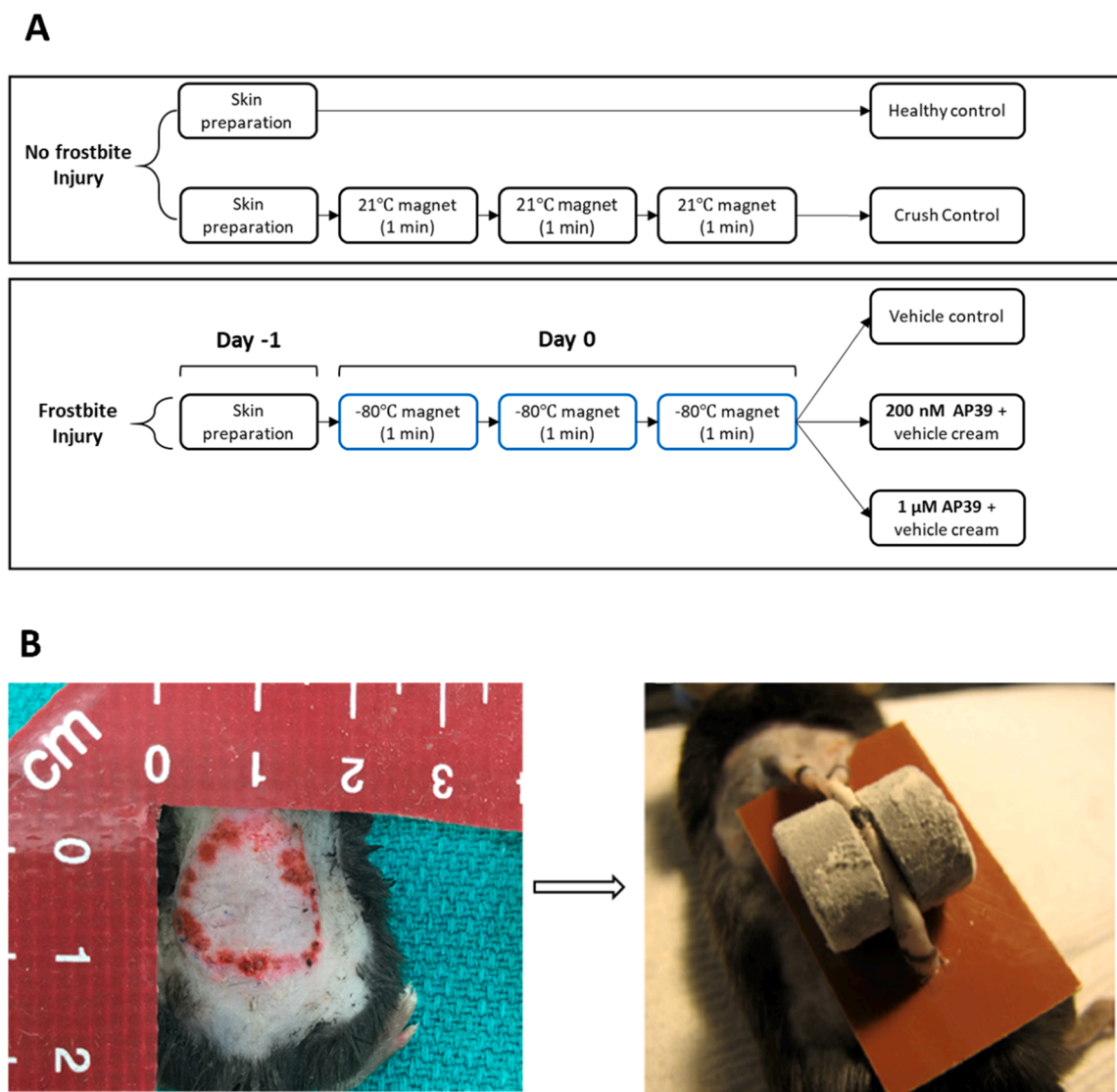


Fig. 1. Experimental design of the acute frostbite model showing (A) flowchart of the experiment and (B) placement of ceramic magnets frozen at -80°C on either side of the dorsal skin to induce frostbite injury after the dorsal skin was shaved.

$^{\circ}\text{C}$ to mitigate the risk of death due to systemic hypothermia. Upon induction of frostbite injury, the mice were randomly assigned to three groups: vehicle control, 200 nM AP39, and 1 μM AP39, and received daily topical application of vehicle cream without AP39, vehicle cream containing 200 nM AP39 (200 μL) and vehicle cream containing 1 μM AP39 (200 μL) respectively until time of sacrifice. Images of the frostbite injury were taken on days 4, 7 and 15 following induction of frostbite injury. The frostbite wound area was measured on days 4 and 15 and quantified using ImageJ version 1.52b software (National Institutes of Health, USA). As AP39 was synthesized in-house [19], we chose these two concentrations (200 nM and 1 μM) due to our previous success with the former concentration in mitigating cold IRI [20] while the latter concentration was based upon our work *in vitro* which suggested a deleterious effect.

2.5. Room temperature magnet placement

To control for possible crush injury created from pressure of the magnets used to induce frostbite, the procedure was repeated in a group of mice that received no frostbite injury but had room temperature magnets (21°C) applied on their dorsal skin (crush control group). Post-procedure analgesia included administration of meloxicam at 1 mg/kg/

day for 3 days. Another group of mice that received no frostbite injury or room temperature magnet placement served as healthy controls. All groups of mice were sacrificed by CO_2 euthanasia on day 15 after frostbite induction or room temperature magnet placement, and samples of the injured dorsal skin tissue were collected and stored in 10 % neutral buffered formalin for histopathological analysis. The animals were euthanized on day 15 because this is the time point at which markers of wound healing were expected to be best seen in the wound as previously reported [21].

2.6. Histopathology and immunofluorescence staining

The formalin-fixed skin tissue samples were embedded in paraffin blocks and sectioned at 8- μm thick on a microtome and collected on SuperFrost Plus® glass slides (J1800AMNZ, Thermo Fisher Scientific Inc., Germany). Histology was performed on the tissue sections using hematoxylin and eosin (H&E) staining to visualize general tissue morphology and assess the degree of tissue injury. A modified version of a previously developed histological scoring system established by Abramov et al. [22] was used for this study. The histopathological scoring was assessed blindly by a dermatopathologist for 5 parameters of healing (acute inflammation, chronic inflammation, amount of

granulation tissue, fibroblast maturation and re-epithelialization) and assigned a score as per the following scheme: 0 = None; 1 = Scant; 2 = Moderate; 3 = Abundant. Acute inflammation was scored based on the presence of neutrophils while chronic inflammation was evaluated based on the presence of plasma cells and monocytes [22]. Fibroblast maturation was determined on the basis of shape and alignment of these cells [22].

Immunofluorescence staining was performed to detect the expression of cleaved caspase-3 (1:100; a marker for apoptosis and is being used to assess frostbite damage), CD31 (1:100; a marker of vascularization and angiogenesis, as it is found on early endothelial cells), KI-67 (1:50; a marker of proliferation, which is abundant in a healing wound as granulation tissue forms and re-epithelialization occurs), fibronectin (1:100; secreted by fibroblasts to generate granulation tissue, which is the new connective tissue that covers a wound.), cytokeratin (1:200; a marker of differentiation and proliferation of keratinocytes, which are important for re-epithelialization of a wound) and CD163 (1:200; a macrophage-specific marker during wound healing) [23,24]. These antibodies were purchased from Abcam in Toronto, Canada to examine the degree of frostbite injury as well as important healing stages including angiogenesis, re-epithelialization, granulation tissue formation and inflammation. Following a rehydration step in xylene and decreasing series of alcohol (100 %, 95 %, 70 % and 50 %) and de-ionized water, the tissue sections were washed with 1X phosphate buffered saline (PBS) for 10 min. The sections were subjected to antigen retrieval with citrate buffer (pH = 6.0) at 95 °C for 10 min after which they were washed with 1X PBS for 5 min and incubated in a blocking buffer (1 % horse serum in PBS) for 30 min at room temperature to block any non-specific staining between primary antibodies and the tissue. The primary antibodies were diluted in 1 % bovine serum albumin (BSA) and 200 μ L of the antibody dilution was added to each tissue slide and incubated overnight at 4 °C to allow for optimal specific binding of the antibodies to tissue targets and reduce non-specific background staining. Following a washing step (3 times with 1X PBS) for 15 min, the tissue sections were incubated in the respective secondary antibodies (diluted in 1 % BSA; 200 μ L per tissue section) for 60 min at room temperature after which the tissue sections were washed again 3 times (with 1X PBS) for 15 min. To detect nuclei signals, 300 μ L of the diluted solution of 40, 6-diamidino-2-phenylindole (DAPI, 1 μ L/mL; D9542, Sigma-Aldrich, Oakville, Canada) was added to each tissue section and incubated in the dark for 2 min at room temperature. The sections were then rinsed once with PBS and mounted with an anti-fade mounting media. The stained tissue sections were visualized and relative fluorescence images of the sections were taken with a wide-field fluorescent microscope (Olympus IX83). Stained pixel areas of the tissues were quantified using ImageJ version 1.52 software (National Institute of Health, USA) and the results were expressed as arbitrary fluorescence unit.

2.7. Statistical analysis

Values are presented as mean $\pm$ standard deviation (SD). Statistical analysis of all data was performed using GraphPad Prism software version 9 (La Jolla, CA). Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Significant difference between groups was indicated with $p < 0.05$.

3. Results

3.1. AP39 enhanced healing of frostbite wound from inflammatory to proliferative phase

As shown in Fig. 2A and B, topical application of both doses of AP39 (200 nM and 1 μ M) enhanced frostbite wound healing as evidenced by progressively and significantly reduced wound area compared to vehicle-treated control mice. Fig. 2C represents histopathological changes on day 15 following induction of frostbite injury and treatment

with and without AP39 as well as injury from room temperature magnet placement (crush control). Compared to healthy control skin, induction of frostbite injury caused regenerative changes with loss of epidermal layer, increased underlying dermal cellular infiltrates and re-epithelialization, while placement of room temperature magnet resulted in thickening of the epidermal layer (Fig. 2C). Quantitatively, acute inflammation was significantly greater in the 1 μ M AP39-treated group than in the 200 nM AP39 and vehicle control groups (Fig. 2D; $p < 0.05$) while chronic inflammation did not change in all three frostbite groups (Fig. 2D; $p > 0.05$). Also, 1 μ M concentration of AP39 markedly increased the amount of granulation tissue compared to that in 200 nM AP39 and vehicle control groups (Fig. 2D; $p < 0.05$). However, fibroblast maturation was highest in 200 nM AP39-treated group in comparison with 1 μ M AP39 and vehicle control groups (Fig. 2D; $p < 0.05$). While AP39 strongly influenced acute inflammation as well as amount of granulation tissue and fibroblast maturation, it also increased wound re-epithelialization (epithelial restitution) albeit not significantly different compared to vehicle control group (Fig. 2D; $p > 0.05$). Collectively, AP39 treatment enhanced frostbite wound healing from inflammatory to proliferative phase characterized by increased formation of granulation tissue, fibroblast maturation and re-epithelialization.

3.2. AP39 promoted angiogenesis, proliferation and granulation tissue formation

We determined the expression of markers of apoptosis (cleaved caspase-3), vascularization and angiogenesis (CD31) and cell proliferation (KI-67) following induction of frostbite injury and injury from room temperature magnet placement. Interestingly, cleaved caspase-3 was abundantly expressed in the wound of mice that received room temperature magnet placement while its expression was surprisingly and significantly reduced in all groups including vehicle control group to the same level with both AP39-treated groups (Fig. 3A–D; $p < 0.01$). Thus, AP39 had no effect on apoptosis in frostbite injury. However, CD31 and KI-67 proteins were markedly expressed in the wounds of 200 nM AP39-treated mice while their expressions were significantly reduced in the wounds of all other groups including 1 μ M AP39-treated and vehicle control groups (Fig. 3A–D; $p < 0.05$). In addition to these markers, we also evaluated the expression of CD163 (M2-like anti-inflammatory macrophages), fibronectin (granulation tissue formation) and cytokeratin (epidermal keratinocyte hyperproliferation). While CD163 expression (Fig. 3A and E) followed the same pattern as observed in Fig. 3A and D, suggesting increased anti-inflammatory activity in the wounds of 200 nM AP39-treated mice, fibronectin was equally expressed in both AP39 groups, with significantly increased expression compared to vehicle control mice (Fig. 3A and F). Finally, cytokeratin expression in both AP39-treated groups significantly decreased while its expression markedly increased in vehicle control mice (Fig. 3A and G; $p < 0.0001$) and crush control groups (Fig. 3A and G; $p < 0.01$). Taken together, topical application of AP39 promoted angiogenesis, cell proliferation and generation of granulation tissue, leading to quick healing of frostbite wound.

4. Discussion

This is the first study to investigate the therapeutic effect of a hydrogen sulfide (H_2S) delivered via a donor molecule, AP39, on frostbite injury. In this study, histological examination and different parameters of wound healing were assessed on day 15 following induction of frostbite injury and treatment with two concentrations of AP39 (200 nM and 1 μ M AP39) in a vehicle cream. These parameters were measured to investigate the efficacy of AP39 in mitigating cold ischemia-reperfusion injury and overall healing of frostbite wound.

As expected, induction of frostbite injury resulted in acute and chronic inflammation. Although the degree of chronic inflammation did not change in all three frostbite groups, acute inflammation was greater

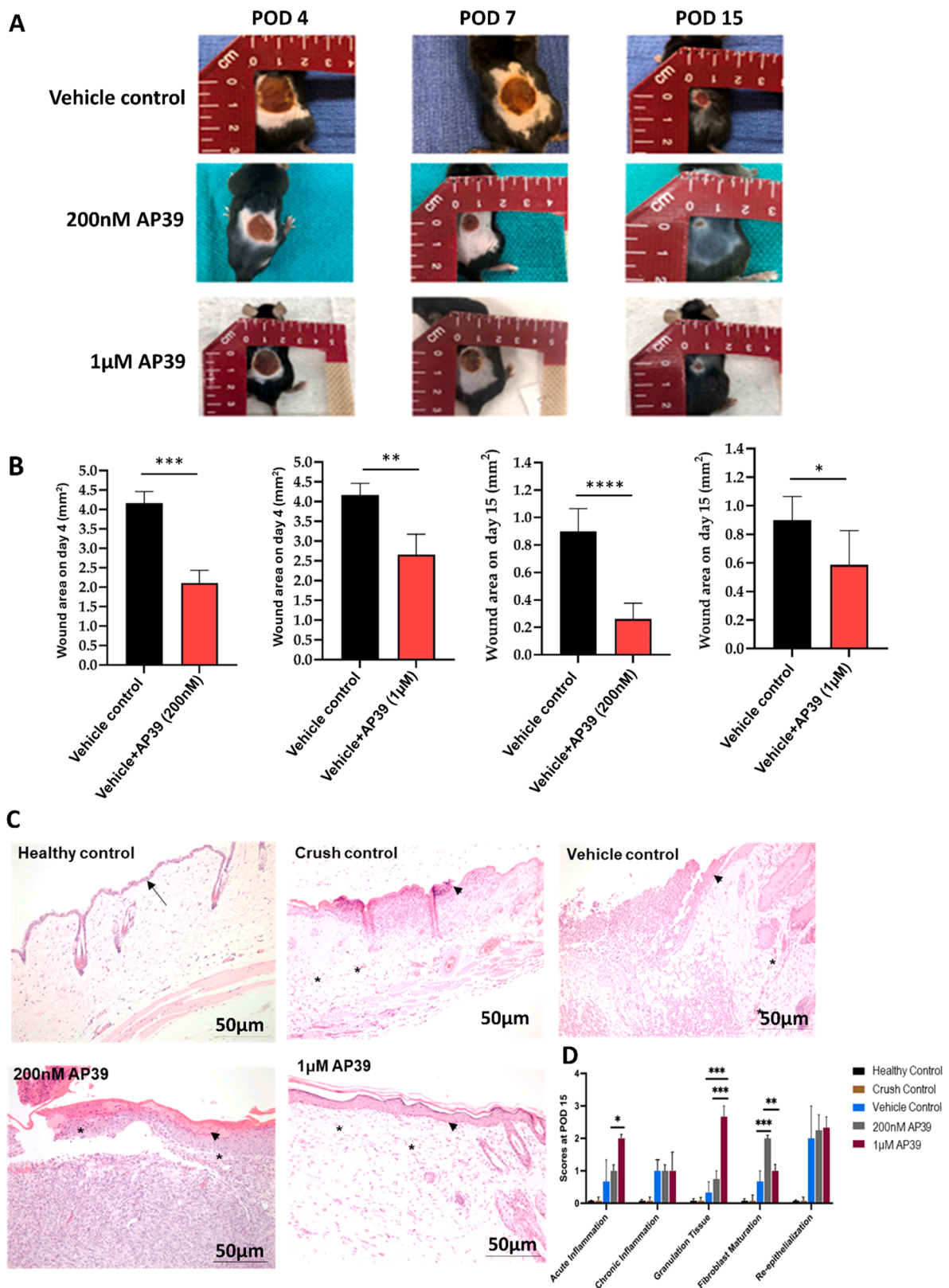


Fig. 2. Frostbite wound and histopathology. (A) Progression of frostbite wound from day 4 to day 15 after induction of frostbite injury. Both concentrations of AP39 enhanced frostbite wound healing and contracture compared to vehicle control. (B) Quantification of wound area on days 4 and 15 after induction of frostbite injury. (C) Histopathological changes on day 15 following induction of frostbite injury and treatment with AP39 showing normal skin with intact epidermal layer (black arrow), regenerative changes with thickening of epidermal layer (arrowhead), and underlying dense dermal cellular infiltrate (*) with dermal fibroblasts. The images of whole wound tissues were taken under at 40x magnification. (D) Quantification of acute and chronic inflammation, granulation tissue formation, fibroblast maturation and re-epithelialization. * $p < 0.05$ vs. vehicle control, ** $p < 0.01$ vs. vehicle control, *** $p < 0.001$ vs. vehicle control, **** $p < 0.0001$ vs. vehicle control.

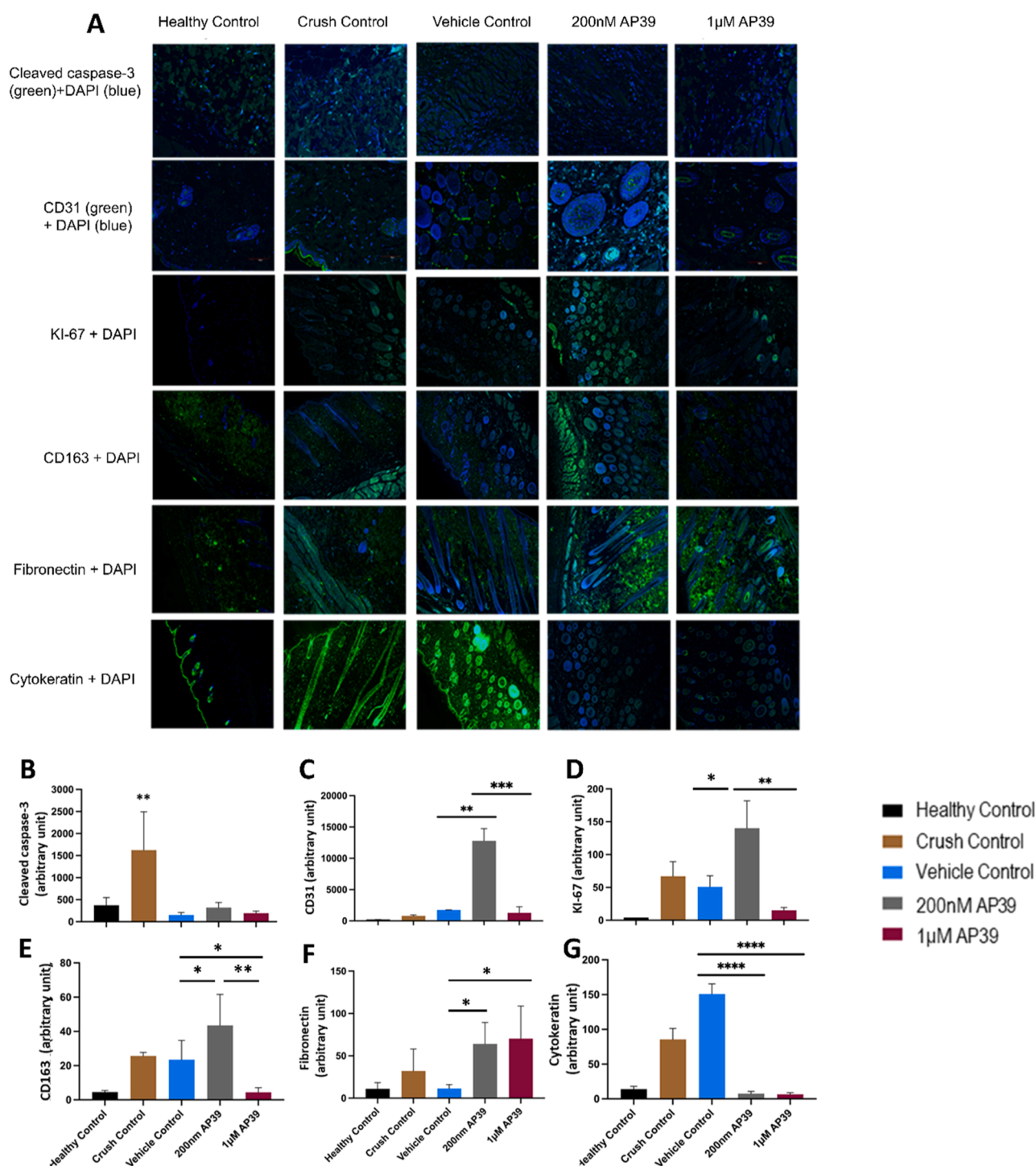


Fig. 3. Immunofluorescence and quantification of markers of wound healing showing (A) cleaved caspase-3, CD31, KI-67, CD163, fibronectin and cytokeratin. Quantification of immunofluorescence indicating the degree of (B) apoptosis, (C) angiogenesis, (D) cell proliferation, (E) anti-inflammatory activity, (F) granulation tissue formation and (G) keratinocyte proliferation. * $p < 0.05$ vs. vehicle control, ** $p < 0.01$ vs. vehicle control, *** $p < 0.001$ vs. vehicle control.

in the wounds of 1 μ M AP39-treated mice. This may suggest that at a concentration of 1 μ M, AP39 triggers influx of immune cells (neutrophils and macrophages), which produce increased levels of inflammatory cytokines and activate fibroblasts to form new tissues in the healing process. Hence, it was not surprising that we observed high expression of fibronectin (primarily secreted by fibroblasts) in the 1 μ M AP39-treated group, which positively correlated with increased formation of granulation tissue and suggests progression from inflammatory phase of wound healing to proliferative and remodelling phases. Thus,

granulation tissue is a measure of wound healing, as cutaneous wounds are known to heal by generating new tissue, which includes both granulation tissue and re-epithelialization [25]. An increased amount of granulation tissue in the AP39-treated groups provides support that treatment of frostbite injury with AP39 has a role in improved tissue healing. Our observation aligns with that of a previous study in which topical application of JK-1 (an H_2S donor) for 15 days increased granulation tissue formation in cutaneous wound of mice [21]. Addition of sodium hydrosulfide (NaHS; another H_2S donor) to nanofibrous

membrane dressings was recently reported to promote wound healing by enhancing granulation tissue formation in a mouse model for cutaneous wound healing [26]. While fibroblast maturation increased in the frostbite wounds of 1 μ M AP39-treated mice, it further increased significantly in the wounds of 200 nM AP39-treated mice. This means that the lower concentration of 200 nM AP39 significantly influenced fibroblast growth to play a crucial role in all the three phases of wound healing (inflammation, proliferative and remodeling), most importantly for dermal restoration. Such restoration by fibroblasts is achieved by enhancing several important cellular developments including deposition of extracellular matrix (ECM), formation of collagen structures to support other cells involved in wound healing and contraction, and remodeling of new ECM. The 200 nM AP39 also exerted anti-inflammatory effects by upregulating the expression of CD163, an M2-like macrophage-specific anti-inflammatory marker that is commonly expressed during wound healing. Our finding is also consistent with that of a recent mouse model for dermal healing in which novel H₂S-releasing hydrogel markedly increased M2-like macrophages and improved wound repair and regeneration [27]. It is important to note that early in wound healing, M1-like pro-inflammatory macrophages in the local macrophage population are recruited into the inflammatory phase, the first stage of wound healing [28,29]. Later as wound healing progresses, these M1-like pro-inflammatory macrophages transition to M2-like anti-inflammatory macrophages to support wound healing [28–30]. M2-like macrophages are an important source of cytokines that stimulate cells involved in wound repair, clear cellular debris, phagocytose apoptotic cells and dampen the inflammatory phase of wound healing [28,30,31]. Thus, the high presence of M2-like anti-inflammatory macrophages revealed by CD163 immunofluorescence staining in the present study indicates transition from inflammatory phase to the next phase of wound healing, and thus contributed to improvement in wound healing.

In addition, the 200 nM AP39 promoted angiogenesis or neovascularization, and cell proliferation as seen in our CD31 and KI-67 immunofluorescence staining (Fig. 3C and D) respectively, which are prominent features of the proliferative phase of wound healing. As blood vessels are severely damaged and clots are increasingly formed in fresh frostbite injury, our result shows that 200 nM AP39 promoted the formation of new blood vessels from the existing ones (neovascularization) in the well-established process that involves invasion of fibrin/fibronectin-rich clot and formation of a microvascular network throughout the granulation tissue [32,33]. Thus, AP39 enhanced the creation of a new vasculature via angiogenesis, which is important in wound healing, as it provides nutritive perfusion at the wound site to regenerating tissues, and without which wound closure and healing will be delayed. Our result supports that of a previous study by Wang and Li [24] who created an ischemic diabetic adductor mouse model and administered H₂S donor sodium bisulfide topically. In this study, the authors demonstrated that H₂S improved angiogenesis in diabetic mice with similar ischemic and vascular damage to our frostbite model. Our result is further supported by a report from recent mouse model of non-healing wound, in which 15 days of topical application of H₂S donor JK-1 resulted in increased angiogenesis [21]. It is important to note that these H₂S donor compounds are non-targeted sources of sulfide and are not the same as AP39. Wu and colleagues [34] also reported increased angiogenesis and improved dermal wound regeneration in animals when novel H₂S-releasing nanofibrous coating was applied during wound dressing. As already mentioned, 200 nM AP39 promoted cell proliferation (increased KI-67 expression), a process which is followed by migration to ensure regeneration of lost tissue. The wound cells that are involved in proliferation and migration include endothelial cells, fibroblasts and keratinocytes. Intriguingly, while AP39 increased fibroblast proliferation for dermal restoration as discussed above, and increased re-epithelialization in our H&E staining, which is similar to the report of Zhao et al. [21] after 15 days of topical application of JK-1H₂S donor, it did not promote proliferation of keratinocytes, a

major cellular component of the epidermis involved in re-epithelialization. This was shown in our cytokeratin immunofluorescence staining. It is possible that AP39 stimulated migration of a host of other cells such as epithelial cells from the surrounding epidermis over the denuded surface to ensure re-epithelialization (epithelial restitution). It is also possible that AP39 might have been metabolised before getting to that layer. Perhaps a higher concentration of AP39 than what we used would increase keratinocyte proliferation, migration and differentiation. While keratinocyte expression was reduced in both AP39-treated groups, the wounds of mice in the vehicle control group exhibited significantly higher cytokeratin expression, which may be attributable to the vehicle cream which contains hyaluronan and phosphatidylethanolamine, both of which have been shown to promote keratinocyte proliferation [18].

An important observation in our study revealed by our caspase-3 immunofluorescence staining is the near absence of apoptosis in the wounds of all three frostbite groups while apoptosis in crush control group was significantly high. While this is surprising, Auerbach and colleagues [2] also did not observe apoptosis in frostbite injury in a mouse model similar to ours. Perhaps apoptosis may have occurred by a caspase-independent mechanism in response to intrinsic apoptotic cues. It is also possible that cell death may have occurred by mechanisms other than apoptosis such as necrosis or cold-induced “bubbling cell death” [35,36].

Although our study provides experimental evidence that AP39 improved healing of frostbite wound, there were several limitations that should be considered in future studies. Firstly, our experimental design did not include frostbite control group without cream application, as the Research Ethics Committee did not approve that group on the ground of “refinement”. It would have been useful to include a group which received frostbite, but without treatment as a baseline for frostbite injury and to separate the effects of the vehicle cream from the effects of the AP39. As the present study is just a pilot investigation, we do not know whether the cream and the AP39 may have had a synergistic or antagonistic effect, or acted independently. Secondly, considering that mitochondria contribute significantly to conditions involving IRI, including frostbite, our study did not examine the impact of AP39 on mitochondrial homeostasis. However, pretreatment of bEnd.3 cells (mouse brain cell line) with low concentrations of AP39 (30–100 nM) has been reported to attenuate glucose oxidase-induced mitochondrial oxidative stress by suppressing mitochondrial ROS production and oxidative protein modification, leading to increased mitochondrial bioenergetics and cell survival [39]. This observation aligns with previous results from rat models of frostbite injury in which induction of frostbite resulted in hydropic degeneration of mitochondria, depletion of energy reserves as well as reduction of energy status and activation of anaerobic metabolism, while calmodulin antagonists (thioridazine and trifluoperazine) preserved mitochondrial function by increasing and restoring ATP production, which was markedly reduced by frostbite [37, 38]. This suggests that AP39 works in a similar fashion as the calmodulin antagonists to preserve mitochondrial integrity during frostbite. Interestingly, a higher concentration of AP39 (300 nM) in the study by Szczesny et al. [39] produced the opposite effect, which supports the concentration-dependent effect of AP39 in our study. Given the potential toxicity associated with triphenylphosphonium cation (the mitochondrial targeting moiety of AP39) [40], the superior therapeutic efficacy of AP39 at lower concentrations as observed in our study and that of the previous study, suggests a possible toxicity of AP39 at higher concentrations. Therefore, future studies should consider investigating the interplay between mitochondrial-targeting property of AP39 and its therapeutic effect, and further investigate its hormetic property in frostbite injury. Lastly, the frostbite wounds we created were confined to the dermis of the mice, and do not represent a true model for deeper frostbite injuries that require amputation or involve significant tissue loss. Future investigations could include animals which have a blistering phase in frostbite wound progression which would closely mimic clinical

version of frostbite wounds and would begin to show whether AP39 treatment would work in humans in the future.

5. Conclusion

To the best of our knowledge, we have provided the first preclinical evidence that topical application of AP39 improves healing of acute frostbite wounds. Given that frostbite injury often occurs during winter activities distant from medical facilities, it would be useful if a frostbite treatment was easy to carry along and administer without specialized medical training. Topically applied AP39 would be an attractive treatment for frostbite in the field, and may potentially help mitigate the significant morbidity associated with frostbite injury. Our data suggest that H₂S may provide a promising avenue for treatment of frostbite injury and could assist in standardizing treatment protocols and improving long-term patient outcomes in the field of regenerative medicine for skin tissue regeneration.

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CRediT authorship contribution statement

Roberta Torregrossa: Resources. **Sally Major:** Project administration. **Patrick McLeod:** Software. **Winnie Liu:** Software. **Aaron Haig:** Software. **Alp Sener:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Lucas N. Penney:** Methodology. **Lauren Mills:** Methodology. **Max Y. Zhang:** Software. **Mark E. Wood:** Resources. **Smriti Juriasingani:** Software. **Matthew Whiteman:** Resources. **Eva Turley:** Resources. **Carl Postenka:** Software. **George J. Dugbartey:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

Matthew Whiteman and the University of Exeter have intellectual property (patent filings) related to hydrogen sulfide delivery molecules, including AP39 and their therapeutic use. The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Matthew Whiteman, Mark E. Wood and Roberta Torregrossa, have patents pending to slow-release sulfide-generating molecules and their therapeutic use. Matthew Whiteman is CSO of MitoRx Therapeutics, Oxford, U.K, developing organelle-targeted molecules for clinical use. All other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Data availability

The data that has been used is in the manuscript

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