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ORIGINAL RESEARCH

Poly-L-Arginine Topical Lotion Tested in a Mouse Model for Frostbite Injury

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Background.—Frostbite injury occurs when exposure to cold results in frozen tissue. We recently reported a novel mouse model for frostbite injury to be used in screening potentially therapeutic drugs and other modalities.

Objective.—We used the mouse skin frostbite model to evaluate the effect of poly-L-arginine contained in lotion (PAL) applied topically to involved skin.

Methods.—Sixty mice were studied in a randomized, double-blind method. Standardized 2.9-cm-diameter circles were tattooed on the mouse dorsum. Magnets snap frozen in dry ice (–78.5°C) were used to create a frostbite injury on skin within the circle as a continuous 5-minute freeze. Mice were treated with prefreeze placebo, postthaw placebo, combined prefreeze and postthaw placebo, prefreeze with PAL, postthaw with PAL, or combined prefreeze and postthaw with PAL. Appearance, healing rate, tissue loss, and histology were recorded until the wounds were healed.

Results.—Application of PAL before inducing frostbite injury resulted in decreased tissue loss as compared with other treatment conditions.

Conclusions.—Applying PAL topically to frostbitten mouse skin caused decreased tissue loss. Poly-L-arginine should be studied further to determine whether it is a beneficial therapeutic modality for frostbite injury.

Key words: frostbite, poly-L-arginine, cold injury, wound healing, mouse model

Introduction

We recently invented a novel mouse skin model of induced frostbite injury, with the intention of using the model to allow quantification of the surface area of involved skin, histology of the wound, rate of wound healing, and skin loss in a standardized fashion after frostbite injury.[1] One purpose for this model is to facilitate efforts to establish or refute efficacy of preventive techniques or therapeutic interventions in a screening manner. To this end, we selected a compound containing poly-L-arginine, which supports nitric oxide synthesis, for testing. Our hypothesis was that we might see a beneficial effect on induced frostbite injury.

Nitric oxide synthase (NOS) is a group of enzymes that helps catalyze production of nitric oxide (NO) from L-arginine, NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate), and molecular oxygen. The signaling molecule NO plays conflicting physiological roles. It helps modulate vascular tone and angiogenesis, but is also a free oxygen radical and can be toxic at high concentrations, particularly in inflammatory disorders.[2] At normal NO levels, there is protection against vascular inflammation and oxidative injury. But when NO rises above physiological levels, it reacts with superoxide anions to produce peroxynitrite anion (ONOO–). This compound combines with lipids, proteins, and DNA to form nitrosoperoxocarbonate, leading to oxidative damage of tissues.[3] Normally not present in resting cells, the inducible form of NOS (iNOS) is part of an immune response induced mainly as a result of the presence of cytokines, producing NO as a defense mechanism. Activation of iNOS raises NO independent of cellular calcium levels, which can have protective or destructive implications.

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L-Arginine is one of the 20 most common amino acids and is manufactured by the body when needed. The amino acid plays a role in cell division and tissue repair, and reduces the healing time of bone injuries.[4] L-Arginine is a substrate for NOS, but is limited inside cells. Poly-L-arginine effectively transfers L-arginine across cell membranes to serve as an intracellular reservoir of L-arginine.[5–9] In an oxidative environment and without sufficient L-arginine, iNOS generates superoxide radicals in a manner that injures endothelium and can cause intimal hyperplasia and smooth muscle accumulation within arteries, which unfavorably thickens their walls, resulting in a form of vasculopathy.[10] With sufficient L-arginine, endothelial NOS produced by vascular tissue generates NO, which inhibits intimal hyperplasia and monocyte adherence and infiltration, slowing smooth muscle cell proliferation and inducing smooth muscle cell apoptosis.[10] Nitric oxide enables veins to strengthen their walls by allowing intimal cells to lengthen and thicken.[11] It also promotes vasodilation by a variety of mechanisms.

Poly-L-arginine also has been shown to penetrate skin and to have the ability to deliver, via covalent attachment, cargo into skin.[12–14] Because reperfusion injury might be a component of frostbite injury and NO can be protective from ischemia–reperfusion injury, we elected to observe the impact on a mouse frostbite injury of topical application of poly-L-arginine contained in a lotion.[15]

Methods

FROSTBITE MODEL

The method of Auerbach et al[1] was used and is briefly recapitulated here. Sixty 8- to 12-week-old C57Bl/6J male mice (Jackson Laboratories, Bar Harbor, ME) were used in a study approved by the Stanford Institutional Review Board and in accordance with Stanford University Institutional Animal Care and Use Committee Guidelines. The animals were housed 5 per cage before and after surgery in a temperature-controlled facility under a 12-hour light/dark cycle.

Skin preparation

Twenty-four hours before day 0, mice were individually anesthetized using isoflurane (Baxter, Deerfield, IL). The dorsal skin surface from the base of the neck to the top of the rear haunches was shaved with an electric clipper, after which a depilatory cream (Nair, Church & Dwight, Princeton, NJ) was applied for 2 minutes to remove any remaining hair. After depilation, the skin was cleansed with a 70% isopropyl alcohol swab. A circle of uniform diameter 2.9 cm was gently traced on the skin with a permanent marker using a silicone sheet template (Invitrogen, Grand Island, NY). The marked circle was then permanently darkened by using an animal tattoo machine (Animal Identification and Marking Systems Inc, Hornell, NY).

Freeze method

Twenty-four hours after skin preparation, ceramic (ferrite) magnets (diameter 0.5 inches, thickness 0.219 inches, weight 3.5 g) were placed in crushed dry ice (–78.5° C) and allowed to freeze for 15 minutes. Using fingers, the back skin of the mouse was lifted into a skin fold and then 2 frozen magnets were placed so that they adhered from opposite sides of the intervening skin fold. Sets of 2 cooled magnets were left in place for 1 minute, then removed to allow new magnets to be immediately placed in the same location against the frozen tissue. The magnet exchange was repeated for a total of 5 applications, resulting in a freeze time slightly longer than 5 minutes. After 5 magnet placements and removals, the skin was allowed to completely thaw. Mice were given subcutaneous injections of buprenorphine (0.05 mg/kg) for analgesia after the thaw. No dressings were applied to the wounds.

Application of poly-L-arginine suspended in AmLactin lotion

One hundred micromolar poly-L-arginine (Lumen Therapeutics, LLC, Menlo Park, CA) was homogenized in 15 mL of AmLactin (Upsher-Smith Laboratories, Maple Grove, MN), a commercially available skin-moisturizing lotion. Placebo lotion was AmLactin absent poly-L-arginine. The mice were divided into 6 groups: poly-L-arginine in AmLactin (PAL) applied prefreeze; PAL applied postfreeze; PAL applied both prefreeze and postfreeze; AmLactin alone applied prefreeze; AmLactin applied postfreeze; and AmLactin applied both prefreeze and postfreeze. For groups consisting of pretreatment only, 1 hour before induced freeze injury 0.5 mL of room temperature AmLactin or PAL was rubbed into the skin with fingers until it was uniformly distributed. For groups receiving posttreatment only, 0.5 mL of room temperature AmLactin or PAL was rubbed into thawing skin with fingers until uniformly distributed. For groups receiving both pretreatment and posttreatment AmLactin or PAL, both actions described above were performed. All mice were monitored until the AmLactin or PAL was visually noted to be completely absorbed.

WOUND SURFACE ANALYSIS AND TISSUE LOSS QUANTIFICATION

Digital photographs of the wounds were taken after the thaw on day 1 and every other day thereafter until day