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Influence of ISDN, L-NAME and selenium on microcirculation, leukocyte endothelium interaction and angiogenesis after frostbite

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ABSTRACT

Background: The body of knowledge regarding the different facets of frostbite injury continues to expand. However, beside the administration of physiological saline, local rewarming, local disinfection and symptomatic medications, today no causal therapy is known which would accelerate angiogenesis and wound healing.

The aim of this study was to investigate the influences of dilative acting drugs on microcirculation, angiogenesis and leukocyte behavior.

Materials and Methods: Ears of male hairless mice ($n = 40$) were inflicted with full thickness frostbites using a cold air jet. Then the affects of four intraperitoneal injections of isosorbitdinitrate (ISDN, $n = 10$), L-nitroarginine-methyl-ester (L-NAME, $n = 10$), selenium ($n = 10$) or sodium chloride ($n = 10$; each administered to one of four corresponding study groups), on microcirculation, leukocyte-endothelial interaction and angiogenesis were investigated over a 12-day period using intravital fluorescent microscopy.

Results: Angiogenesis was most improved by ISDN (36.8 vs. 54.5% non-perfused area on day 3, 3.9 vs. 17.0% on day 7 compared to selenium, $p < 0.006$). Venular diameter was most significantly dilated in the ISDN-group, L-NAME showed significantly decreased diameter over the complete time of 12 days. ISDN had positive influences on edema formation, which was significantly reduced compared to control (27% lower values compared to control; $p = 0.007$ on day 3). The L-NAME-group showed the significant highest leukocyte-adhesion compared to control on days 7 and 12 (53% resp. 58% higher, $p < 0.006$).

Conclusion: Overall, out of all the drugs tested, ISDN improved angiogenesis, dilated venules and decreased edema formation and therefore seems to have the greatest positive impact on these crucial parameters after frostbite injury.

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1. Introduction

Today, a high prevalence of severe cold damage can nowadays be found in the general population [1], work-related and among homeless people [2], whereas in the past mostly people in military environments were affected [3]. Especially an increasing tendency to extreme outdoor activities and a rising number of diabetes increases the frequency of severe frostbites [4]. The death rate in the United States is 0.2 per 100,000 population a year [5].

The tissue response is generally dependent on the degree of cooling [6]. Under colder conditions an extra- and intracellular ice crystal formation occurs, resulting in edema formation, malfunction of the enzymes and cell death [5,7].

Two damaging components of frostbites are known. The first one is the described freeze injury itself. The second one is the reperfusion-damage during rewarming [5]. Red cells, platelets and leukocytes in interaction with the injured endothelium cause microvascular thrombosis [1,8,9]. Additionally, free radicals and arachidonic acid metabolites, like PGF_{2a} and thromboxane A_2, are released, further aggravating vasoconstriction and thrombosis [1,10].

No existential improvements in the treatment of patients with frostbites have been achieved during the last 25 years [11]. Several treatment modalities to improve blood flow by thrombolysis and vasodilatation or to reduce edema were tested. However, the majority of research studies has not been followed up or was performed without adequate controls [1,12–15].

Therefore, we hypothesized that the potent vasodilator isosorbitdinitrate respectively the Nitric oxide, as a functional group of ISDN, dilate the microvessels and improve wound repair by means of a better blood supply, higher blood flow and therefore less adhesion of leukocytes, causing less reperfusion damage and less microthrombosis. By applying the nitric oxide inhibitor L-nitroarginine-methyl-ester we wanted to prove the opposite effects and learn more about the pathophysiological relationships. We expected less vasodilation and a more pronounced leukocyte adhesion due to slower blood flow. Selenium was used since it is known for its anti-inflammatory and anti-oxidant effects. We hoped to reduce the reperfusion damage, reduce the leukocyte-activity and accelerate angiogenesis and wound healing in general.

The aim of this study was to use a well established and reliable frostbite model to investigate the impact of the vasoactive drugs isosorbitdinitrate (ISDN), L-nitroarginine-methyl-ester (L-NAME) and selenium on microcirculation, leukocyte-endothelium-interaction and angiogenesis, to prove a possible influence and to find out more about the underlying pathophysiological mechanisms. The doses we applied in the present study were chosen according to the recommendations provided by the manufacturer.

2. Materials and methods

Animals: 40 male hairless SKH-1/hr mice (4 Groups, $n = 10$ in each group; bodyweight 20–23 g) were obtained from Charles River, Sulzfeld, Germany. They had access to standard laboratory food and tap water ad libitum. Each procedure was approved by the regional authorities according to German animal care regulations, which comply with the international guidelines of animal care and use in scientific experiments.

Preparations: Mice were anesthetized by spontaneous inhalation of isoflurane-N_2O (F_iO_2 0.35, 0.015 L/L isoflurane, Forene®, Abbott GmbH, Wiesbaden, Germany) and placed on a heated acryl-glass observation platform to maintain body temperature. Subsequently, two microsurgical loops (11/0 Surgipro, Covidien Deutschland GmbH, Neustadt (Donau), Germany) were pulled through the ear to extend it. Physiological saline between the ear and the platform flattened the ear by adhesion.

Frostbite: A deep frostbite was caused by a nitrogen vapour, which was poured out of the catheter orifice at a temperature of 195.8 ± 2.7 °C below zero with a distance of 3 mm to the ear's surface for 2 s, which has been described in detail elsewhere [16].

Intravital fluorescence microscopy: Before induction of the frostbite, fluorochromes were administered via the tail-veins (tube 29G, Braun, Melsungen, Germany). 25 μl FITC labeled dextran (1.0%, MW 150 kDa) served as plasmamarker, and 25 μl rhodamine 6G (0.5%) for staining leukocytes (Sigma Chemicals Co., St. Louis, MO, USA).

The prospective frostbite area was designated and the microcirculatory parameters of the regions of interest (ROI) were assessed prior to the infliction of the frostbite. For the intravital fluorescent microscopy (IFM; Axiotech Vario, Carl Zeiss, Oberkochen, Germany) we used a 4-fold objective for the overview and a 10- and 20-fold water immersion objective (Achroplan, Zeiss, Oberkochen, Germany) to measure the non-perfused area, the microcirculatory measurements and leukocyte–endothelial interaction.

Images were recorded using a charge-coupled video camera (AVT-BC 71, AVT-Horn, Aalen, Germany) and stored digitally. Microscopic observations were performed directly prior to the induction of the injury as well as during frostbite healing for the following 12 days (days 1, 3, 7 and 12).

Drugs: The drugs were injected intraperitoneally 30 min post trauma and on days 1, 2, 3, 4 and 5 after intravital fluorescent microscopy. All drugs were dissolved in sodium chloride in a volume of 250 μl. The four groups include isosorbitdinitrate (ISDN, 250 μl, 8 mg/kg; $n = 10$; Isoket®, UCB Schwarz Pharma, Monheim, Germany), L-nitroarginine-methyl-ester (250 μl, 3 mg/kg; $n = 10$; L-NAME hydrochloride, Sigma–Aldrich Chemie GmbH, Munich, Germany), selenium (250 μl, 60 μg/kg; $n = 10$; Selenase®, Biosyn Arzneimittel GmbH, Fellbach, Germany) and sodium chloride (250 μl, $n = 10$; Natriumchlorid, B.Braun Melsungen, Melsungen, Germany).

Isosorbitdinitrate is its active metabolite Nitric oxide (NO), one of the strongest vasodilator known. Nitroarginine-methyl-ester is a NO-inhibitor and should therefore cause a vasoconstriction or at least a less pronounced vasodilation after frostbite. Selenium is known for its anti-inflammatory and anti-oxidant effects, the exact mechanism of action is not known until now.

Measurements: The microcirculatory parameters were quantified with an off-line computer-assisted image analysis system (CapImage®; Zeintl Software; Heidelberg, Germany). The non-perfused area was recorded and determined using a