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The effects of hydrogen sulfide on microvascular circulation in the axial pattern flap ear model in hairless mice

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ABSTRACT

Background/purpose: Improval of flap viability is one of the main purposes in reconstructive surgery. The endogenous volatile mediator hydrogen sulfide (H_2S) exerts plenty beneficial effects on blood rheology and protects from ischemia reperfusion injury.
Methods: Therefore this study analyzed whether H_2S affects tissue necrosis and skin microcirculation during impaired perfusion in the axial pattern ear flap model in hairless mice. Necrosis and microcirculatory parameters were assessed by means of stereo- and intravital fluorescence microscopy before and immediately after flap creation as well as on days 2 and 4 after surgery. Treatment with H_2S donor GYY4137 or its vehicle was performed either one-time after flap preparation or two-times with an additional injection on day 2. After 4 days ear tissues were taken and further analyzed histologically to study tissue edema.
Results: Compared to vehicle treatment, both one- and two-time treatment with GYY4137 significantly reduced tissue necrosis and improved microcirculation in the ear flap, apparently through capillary dilatation and increased functional capillary density. Histological analysis revealed a significantly reduced tissue edema formation in GYY4137-treated animals. While microcirculatory parameters in parts significantly improved by two-time GYY4137 treatment, ear thickness and edema formation were independent from treatment regime.
Conclusions: In summary H_2S treatment is a promising attempt to improve microcirculation in critically perfused skin flaps and consequently to preserve flap viability.

1. Introduction

One major problem in reconstructive surgery is the skin flap necrosis (Losken et al., 2008). To prevent tissue necrosis an adequate perfusion of the skin flap is essential, including sufficient blood flow from arteries through capillaries to draining venules (Barker et al., 1989a). Therefore both an intact macro- and microcirculation are crucial to assure the viability of a skin flap. Impaired flap perfusion and microcirculation, leading to tissue necrosis, are frequent causes of ischemia reperfusion injury (Kerrigan, 1983; Rücker et al., 1999).

In the last years the endogenous volatile mediator hydrogen sulfide (H_2S) was found to have a pleiotropic character with multiple beneficial effects on the cardiovascular system (Wang et al., 2016a). H_2S is known for protection from ischemia reperfusion injury, which was shown for neuronal (Chen et al., 2016), cardial (Karwi et al., 2017; Wang et al., 2016c), renal (Cao and Bian, 2016; Feliers et al., 2016) and other tissues.

H_2S also exerts anti-hypertensive effects (Brampton and Aaronson, 2016; van Goor et al., 2016), prevents thrombosis by affecting platelet activation (Grambow et al., 2014; Emerson, 2015; Wang et al., 2016b), accelerates thrombolysis (Grambow et al., 2017) and therefore positively affects blood rheology.

In peripheral vascular tissue H_2S is synthesized by cystathionine γ-lyase and 3-mercaptopyruvate sulphurtransferase (Wang, 2012). Both enzymes have further been detected in human skin and it was shown that the synthesized H_2S modulates vascular functions in human cutaneous circulation (Kutz et al., 2015) making the gaseous mediator a potential treatment for patients with vascular dysfunctions.

Potential beneficial effects of H_2S for preservation of cutaneous flap viability in reconstructive surgery were recently shown in a porcine model of vascularized composite autotransplantation. Here H_2S was found to reduce systemic biomarkers for skeletal muscle tissue injury,

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i.e. creatine kinase, lactate dehydrogenase, aspartate transaminase, interleukin-6 and tumor necrosis factor-α, and therefore mitigated reperfusion injury (Villamaria et al., 2014).

With respect to these findings it was the aim of this study to investigate whether treatment with a slow releasing H_2S donor would affect flap necrosis and to characterize the effects of H_2S on skin microcirculation in an established flap model in detail as a potential tool to preserve flap viability.

2. Materials and methods

All in vivo experiments (7221.3-1-022/16) were conducted in accordance with the German legislation on protection of animals and the NIH Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council) and were performed in accordance with the recommendations of Good Laboratory Practices. The animals were housed in a specific pathogen-free facility with a 12 h light-dark cycle and had access to standard laboratory chow and water ad libitum. To study the effects of H_2S on skin microcirculation during ischemic conditions, the axial pattern flap model of the ear of hairless SKH-1/hr mice was used like previously described (Barker et al., 1989a, 1989b; Kubulus et al., 2005; Goertz et al., 2009; Harder et al., 2010).

In short, mice age between 8 and 10 weeks with a body weight (bw) between 25 and 30 g were anesthetized by an intraperitoneal (ip) injection of ketamine (90 mg/kg bw) and xylazine (25 mg/kg bw) and placed on a plexiglas pad with an integrated heating plate for maintaining the body temperature at 37 °C. To create an axial pattern flap the ear was first extended on the plexiglas pad, followed by incision of its caudal base for four fifths. As a result the created ear flap was supplied only by the cranial neurovascular bundle. During the observation time of 4 days animals were orally treated with metamizol (5 drops in 100 ml water from a 500 mg/ml stock solution) to assure analgesia.

The experimental setting (Fig. 1) started with a baseline analysis at day -2 to assess the unimpaired microcirculation using an intravital fluorescence microscope (IVM, Axiotech vario, Zeiss, Jena, Germany) equipped with a 100 W mercury lamp and a blue filter set (465–95 nm/N505 nm excitation/emission wavelength). IVM was conducted upon intravenous (iv) injection of 0.05 ml 5% fluorescein isothiocyanate-labeled dextran (FITC-dextran, MW 150 kDa, Sigma Aldrich, St. Louis, MO) in the tail vein of the mice.

Analysis included assessment of diameter and red blood cell velocity of the principal arterioles and venules in the respective regions as well as assessment of capillary diameter and functional capillary density in

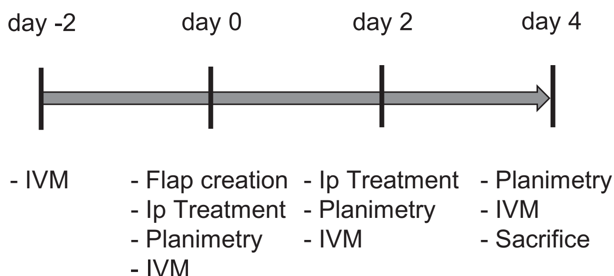

Fig. 1. Time course of the experimental setting. At day -2 intravital fluorescence microscopy (IVM) was performed to assess microcirculatory baseline parameters within the ear. On day 0 the ear flap was created by incision of its caudal base of the ear for four fifths, immediately followed by ip treatment with either GYY or its vehicle and subsequent planimetric and IVM assessment. On days 2 and 4 both planimetric and IVM analysis were performed to assess skin necrosis and microcirculatory parameters. In a separate set of animals GYY treatment was repeated on day 2. All animals were sacrificed on day 4 after planimetric and IVM analysis and the ear tissues were taken for histological analysis.

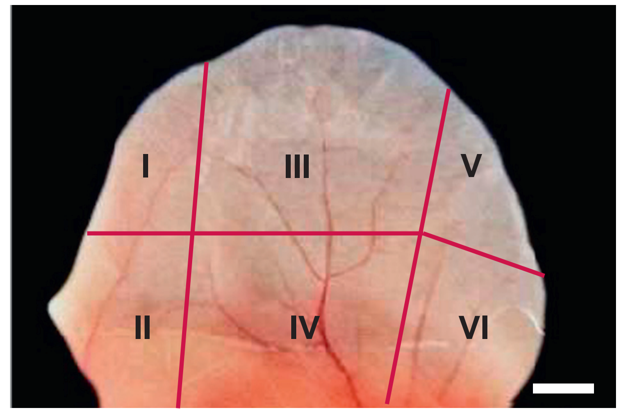

Fig. 2. Photomacroscopic image of an ear flap in a hairless mouse with schematic illustration of the 3 areas that were defined referring to the principal arterio-venular bundles and their division in a proximal and distal part. So the flap was divided in areas I to VI that were assessed separately by means of IVM for repetitive analysis of microcirculatory parameters at the same location. Bar represents 1.25 mm.

two capillary beds that were selected due to their position to the branches of the principal vessels. This made it easy to relocate the capillary beds even when ischemia and perfusion failure lead to formation of edema within the ear tissue that impeded IVM. Both capillary diameter and functional capillary density were assessed in up to five capillaries per capillary bed, allowing reliable evaluation on the effects of H_2S on both parameters.

To standardize IVM analysis of the microcirculation, the ear flap was divided in 3 regions that were delimited by the principal vessels that enter the ear at its base and lead to its periphery (Barker et al., 1989a, 1989b). For a more detailed microvascular analysis each region was further divided in half in a proximal part, ranging from the base of the ear flap to its midline and a distal part, ranging from the midline to the apical edge of the auricle. The defined areas I-VI (Fig. 2) were analyzed separately. Analysis of all parameters was performed offline using the CapImage® software (Klyscz et al., 1997).

On day 0 the axial-pattern flap was created as described above. Subsequently, animals were ip treated with either the H_2S donor GYY4137 (GYY, 50 mg/kg bw, Enzo Life Sciences GmbH, Lörrach, Germany, $n = 7$, Li et al., 2008), dissolved in 30% DMSO, or an equimolar volume of its vehicle DMSO (30% v/v in 0.9% NaCl, $n = 7$).

Afterwards, planimetric assessment of the ear flap was performed using a stereomicroscope (IC-A, Leica Microsystems GmbH, Wetzlar, Germany), followed by IVM as described above. Both stereomicroscopy and IVM were repeated same wise on days 2 and 4 to assess and quantify necrosis and microcirculation of the ear flap respectively.

In a separate set of animals an additional ip injection with GYY (50 mg/kg bw) was performed on day 2 to test the effect of a repetitive treatment ($n = 7$).

On day 4 animals were sacrificed by an overdose of anesthesia and the ear tissues were taken and fixed in 4% phosphate-buffered formalin for 3 days and then embedded in paraffin. From the paraffin-embedded tissue blocks, 4 μm sections were serially cut and counterstained with hematoxylin-eosin for assessment of routine histology and to quantify the extent of edema within the central part of the ear flap ($n = 7$ per treatment). For this purpose, representative images (Fig. 3A) were taken using the cellSens software (Olympus, Shinjuku, Japan). As an indirect parameter for tissue swelling due to edema, the thickness of the ear flap was calculated from 3 independent measurements at different parts of the ear in the central part of the flap over a distance of 2 mm (Fig. 3B). As previously described (Harder et al., 2005b), edema within the muscle and the soft tissue was defined as the intercellular space (Fig. 3C and D), which was quantified by means of the CapImage® software (Klyscz et al., 1997). A random sample of an ear image was

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