Hemoglobin vesicles improve wound healing and tissue survival in critically ischemic skin in mice

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Submitted 7 May 2009; accepted in final form 29 June 2009

Am J Physiol Heart Circ Physiol 297: H905–H910, 2009. First published July 2, 2009; doi:10.1152/ajpheart.00430.2009—Local hypoxia, as due to trauma, surgery, or arterial occlusive disease, may severely jeopardize the survival of the affected tissue and its wound-healing capacity. Initially developed to replace blood transfusions, artificial oxygen carriers have emerged as oxygen therapeutics in such conditions. The aim of this study was to target primary wound healing and survival in critically ischemic skin by the systemic application of left-shifted liposomal hemoglobin vesicles (HbVs). This was tested in bilateral, cranially based dorsal skin flaps in mice treated with a HbV solution with an oxygen affinity that was increased to a P50 (partial oxygen tension at which the hemoglobin becomes 50% saturated with oxygen) of 9 mmHg. Twenty percent of the total blood volume of the HbV solution was injected immediately and 24 h after surgery. On the first postoperative day, oxygen saturation in the critically ischemic middle flap portions was increased from 23% (untreated control) to 39% in the HbV-treated animals (P < 0.05). Six days postoperatively, flap tissue survival was increased from 33% (control) to 57% (P < 0.01) and primary healing of the ischemic wound margins from 6.6 to 12.7 mm (P < 0.05) after HbV injection. In addition, higher capillary counts and endothelial nitric oxide synthase expression (both P < 0.01) were found in the immunostained flap tissue. We conclude that left-shifted HbVs may ameliorate the survival and primary wound healing in critically ischemic skin, possibly mediated by endothelial nitric oxide synthase-induced neovascularization.

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hemoglobin vesicles (HbVs) are known to be part of the plasma (20), the pharmacokinetic behavior of the vesicles, a sulfonrhodamine B solution (10 mM, Invitrogen, Tokyo, Japan) was encapsulated instead of Hb. The two types of vesicles featured otherwise identical physicochemical properties.

**Animals, experimental groups, and flap preparation.** The experiments were performed according to the National Institutes of Health guidelines for the care and use of laboratory animals and with the approval of the local Animal Ethics Committee. Forty-eight DDY mice weighing 22 to 26 g were included in this study. Eighteen animals were used for assessing the pharmacokinetics and plasma viscosity; the others were randomly assigned and equally distributed to an untreated control group and to groups receiving either 0.9% NaCl or HbVs.

The anesthetized animals were placed on a heating pad in a prone position, and the room temperature was set at 28°C to keep the animal’s skin temperature constant at 32°C, which was verified with a microthermometer placed on the abdominal skin. The back skin was shaved and epilated. Surgery was performed with the aid of an operating microscope at ×10 magnification (Wild, Heerbrugg, Switzerland). Two cranially based flaps measuring 30 × 15 mm were dissected on both sides of the dorsal midline (Fig. 1). The flaps consisted of skin, a thin layer of panniculus carnosus muscle, and subcutaneous tissue. The lateral thoracic artery was ligated, thus creating random collateral arterial perfusion of the flap via the subdermal vascular plexus. During surgery, the flap was irrigated with 0.9% NaCl to prevent it from drying out. The flaps were sutured back into their original position after having placed a silicone sheet underneath to avoid vascular ingrowth from the wound bed.

**Tissue oxygenation.** Cutaneous oxygen saturation was measured with a noninvasive microprobe system performing white light spectrometry for computed data collection (O2C-system, LEA-Medizintechnik, Giessen, Germany). According to the manufacturer, the sample surface and depth were ~1 mm² and 800 μm, respectively. Probes were placed on the neck (normal skin) and in the central flap axis at distances of 5 (proximal flap), 15 (middle flap), and 25 mm (distal flap) from the base of the flap.

**Tissue survival and primary wound healing.** The length of the healed wound suture was measured in the midline (ischemic flap tissue sutured to each other) and laterally (ischemic flap tissue sutured to healthy tissue). A digital photographic picture was taken of both flaps, which was computerized for planimetry to calculate the percentage of the total flap surface that became necrotic (Adobe Photoshop, Adobe, San Jose, CA).

**Histological examination.** Tissue samples were taken from the spleen, liver, and kidney, which are where the HbVs are degraded by the reticuloendothelial system and excreted (20). In addition, specimens were harvested from the flap tissue at a standardized distance to the flap basis of 5 mm. This region was chosen to obtain healthy flap tissue well off the inflamed demarcation zone or necrosis. The samples were fixed in 4% paraformaldehyde, washed in PBS, stored in 70% ethanol, and finally embedded in paraffin blocks. Sections (5 μm) were cut, transferred to microslides, and air dried at 37°C overnight. Hematoxylin-eosin staining was performed for morphological evaluation and Giemsa staining for visualizing leukocytes. In addition, the flap tissue specimens were immunohistochemically stained for eNOS. To this end, peroxidase was blocked with 3% H₂O₂, and nonspecific reactions were reduced by protein blocking (DakoCytomation, Protein Block, Serum-Free, Zug, Switzerland). The slides were then incubated with a primary rabbit anti-eNOS antibody (1:50; Santa Cruz, Heidelberg, Germany). The anti-rabbit EnVision Detection System (DakoCytomation EnVision+ System Labelled Polymer-HRP Anti-Rabbit K4003) and AEC (Aminoethyl Carbazole Substrate Kit, Zymed, San Francisco, CA) were used as secondary antibody and chromogen, respectively. Positive and negative controls were taken for each set of antibody and between each step. Immunostaining was assessed semiquantitatively by using light microscopy at a ×200 magnification (Leica DM7RB, Wetzlar, Germany). The total numbers of capillaries and eNOS positive capillaries within one visual field were counted. Data were calculated from the averages of three randomly selected visual fields per specimen.

**Laboratory analysis.** Hb and methemoglobin (met-Hb) concentrations were analyzed in the blood samples (ABL 625; Radiometer, Copenhagen, Denmark). The samples were centrifuged with 3,000 rounds/min for 10 min to determine hematocrit and to obtain the plasma. Since the HbVs are known to be part of the plasma (20), the

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**Fig. 1.** Experimental model. **A and B:** a cranially based skin flap is raised on both sides of the dorsal midline of anesthetized mice. The flap extends the vascular territory of the nourishing lateral thoracic artery and vein (marked in red in A). The lateral thoracic artery is ligated. **C:** the flap is sutured back into its original position after interpositioning a silicone sheet to avoid revascularization from the wound bed. **D:** 6 days postoperatively, a clear demarcation can be observed between vital and necrotic flap skin and between healed and nonhealed suture lines.
supernatant was collected for measuring HbV-bound Hb and met-Hb concentrations separately.

To assess the pharmacokinetics and plasma viscosity, the plasma of three animals was pooled for each time point. Plasma viscosity was measured with a Hôppler-type viscosimeter (HAAKE Messtechnik, Karlsruhe, Germany) at 37°C, and the average of four consecutive measurements was calculated. The plasma concentration of the vesicles was measured with a computerized fluorescence reader (Infinite M 200, Tecan, Maennedorf, Switzerland). For this purpose, the plasma was transferred to 96-well plates. A calibration curve was previously established to transfer fluorescence intensity to vesicle concentration, and native plasma was taken as a negative control.

**Protocol.** For all manipulations, the animals were anesthetized with intraperitoneal injection of medetomidine (500 μg/kg body wt; Dromitor, Pfizer, Zurich, Switzerland), climaZolam (5 mg/kg; Climasol, Graub, Bern, Switzerland), and fentanyl (50 μg/kg; Fentanyl-Janssen, Janssen-Cilag, Baar, Switzerland), and anesthesia was antagonized by injecting the antidotes atipamezole (1.25 mg/kg body wt; Antisedan, Pfizer), sarmazenil (0.5 mg/kg body wt; Sarmasol, Graub), and naloxone (0.6 mg/kg; Naloxon, Orpha, Kusnacht, Switzerland) after the manipulations were completed. Animals were euthanized with an overdose of pentobarbital sodium at the end of the experiments.

Immediately after the flap preparation was completed, 20% of the estimated total blood volume of the HbV solution or 0.9% NaCl was injected into the tail vein over a period of 10 min.

On postoperative day 1, the cutaneous oxygen saturation measurements were taken, and another 20% of the estimated total blood volume of 0.9% NaCl or the HbV solution was injected.

On day 6, tissue survival and primary wound healing were assessed. Thereafter, blood samples were obtained by cardiac puncture for laboratory analysis, and tissue samples were collected for histological examination.

Perioperative death, wound infection, and technical complications such as rupture of the skin suture, thrombosis of the draining vein (leading to total flap loss), or failing arterial ligature (leading to total flap survival) were taken as exclusion criteria.

The same injection protocol was used to determine the pharmacokinetics of the vesicles and the plasma viscosity. Blood samples were obtained by exsanguinating the animals at baseline and 30 min, 24 h, and 48 h after each vesicle solution injection.

**Statistical analysis.** The InStat version 3.0 software (GraphPad, San Diego, CA) was used for statistical analysis. The data are presented as means ± SD. Differences between the groups or sites of measurements were assessed by unpaired analysis of variance and Bonferroni posttest. A value of \( P < 0.05 \) was taken to represent statistical significance.

**RESULTS**

Five animals fulfilled the exclusion criteria (1 death, 1 infection, and 3 technical problems), thus resulting in final sample sizes of \( n = 9 \) in the control group and \( n = 8 \) in each other group in the flap experiments.

On postoperative day 1, we observed a gradual decrease in oxygen saturation from 49% to 11% in the distal parts of the flap in all animals (Fig. 2). Whereas similar values were obtained after NaCl injection, higher oxygen saturation was maintained after HbV in all parts of the flap, which became most evident in the middle flap portion (\( P < 0.05 \)).

![Fig. 2. Oxygen saturation (SO2) in normal skin and proximal, middle, and distal flap skin in untreated control animals and animals receiving 2 × 20% of total blood volume (TBV) of 0.9% NaCl or hemoglobin vesicle (HbV) solution. Data are expressed in mean values and SD. *P < 0.05 and **P < 0.01 vs. normal skin; #P < 0.05 vs. control.](http://ajpheart.physiology.org/)

Fig. 3. Length of primarily healed wounds in midline and lateral suture in untreated control animals and animals receiving 2 × 20% of TBV of 0.9% NaCl or HbV solution. Data are expressed in mean values and SD. \#P < 0.05 vs. control.

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![Fig. 4. Flap tissue survival in untreated control animals and animals receiving 2 × 20% of TBV of 0.9% NaCl or HbV solution. Data represent percentages of total flap surface and are expressed in mean values and SD. ##P < 0.01 vs. control.](http://ajpheart.physiology.org/)
Six days postoperatively, a clear distinction could be made between the healed and nonhealed suture lines, and the demarcation between necrotic and surviving flap tissue was unequivocally discernible (Fig. 1). In the control group, the proximal 6.6 ± 6.5 mm of the midline suture were healed (Fig. 3). This length was increased to 12.7 ± 7.2 mm in the animals having received HbV (P < 0.05), whereas it was virtually unchanged in the NaCl group. The mean length of the primarily healed lateral wound ranged between 10.1 and 11.4 mm and was similar in all groups. Flap tissue survival was increased from 33 ± 20% in the control group to 57 ± 16% after HbV (P < 0.01) but was similar to control in the NaCl group (Fig. 4).

The immunohistochemical assessment revealed 3.0 ± 0.9 capillaries/visual field in the proximal flap tissue of untreated animals, whereas capillary density was raised to 5.3 ± 0.9 capillaries/visual field after HbV (P < 0.01, Figs. 5 and 6). In addition, higher eNOS expression was found in the capillaries of animals treated with the HbV solution, yielding 4.1 ± 1.3 eNOS + capillaries/visual field (P < 0.01). The histomorphological examination of the parenchymal tissues exhibited no signs of organ failure, tissue damage (hematoxylin-eosin staining), or leukocyte accumulation (Giemsa staining) in the liver, spleen, and kidney throughout the groups.

Laboratory blood work revealed similar hematocrit levels (means, 41.8% to 44.0%), Hb concentrations (means, 14.1 to 15.0 g/dl), and met-Hb concentrations (means, 0.02% to 0.04%) in all groups 6 days after flap surgery and 5 days after the last injection. No Hb was found in the plasma phase in any animal. The pharmacokinetic experiments revealed that 68% of the initially injected vesicles were still circulating after 24 h. The initial concentrations (88% and 32%) were found 24 and 48 h after the second injection, respectively (Fig. 7). Plasma viscosity was 1.19 cP in the untreated animals, and 1.41 and 1.31 cP at 24 and 48 h after the second vesicle injection, respectively.

**DISCUSSION**

The principal findings of the present study were that both the primary healing of the wound between the two ischemic, hypoxic skin margins and the survival of critically ischemic tissue were improved after the administration of left-shifted HbVs.

This biological behavior was paralleled by the pattern of oxygen saturation on the first day after surgery, suggesting that
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Tissue oxygenation was sufficient for survival but not for wound healing in the more proximal parts, whereas the oxygen debt reached a severity that was no longer compatible with cell survival more distally. An increased oxygen demand is necessary for normal tissue repair, which is not sufficiently covered under hypoxic conditions, and wound healing may be attenuated due to the suppression of essential oxygen-dependent processes such as fibroblast and myofibroblast activity, angiogenesis, and collagen synthesis (2, 9). An ameliorated oxygen supply was most likely responsible for the improved midline suture healing in the HBV-treated animals in our study. No such effect was observed in the lateral wound margins where tissue repair appeared to be in the domain of the healthy, normoxic skin, not affected by the HBVs. The length of the lateral, primarily healed suture line corresponded to the length of the healed midline suture line in the animals receiving HBV, thus emphasizing the therapeutic potential of this oxygen carrier.

The immunohistochemical analysis yielded significantly higher capillary counts and eNOS expression in the flap tissue of animals receiving HBVs. eNOS exerts a predominant function in angiogenesis and vasculogenesis (7). Besides stimulating new vessel formation, it attenuates endothelial cell apoptosis (1, 15). eNOS is therefore a pivotal player in wound healing (3, 14) and contributes to maintain or improve flap tissue survival (11, 13). eNOS expression is oxygen dependent (12, 16, 23), stimulated by fluid shear stress (15, 24), and inhibited by proinflammatory cytokines (1, 23, 27).

Each of these pathways may have been influenced by the HBVs. With the assumption that the NaCl and H2O components of the administered solutions exited the vascular compartment within a few minutes, the administered dosage of HBVs enhanced the HBV concentration in the whole blood by ~15% after the first injection, and according to the pharmacokinetic experiments, one third of the initially injected amount of HBVs was still circulating 3 days later. Much more importantly than increasing the oxygen carrying capacity, the HBVs regulate oxygen release from the red blood cells to the tissue due to their presence in the plasma phase. If the oxygen affinity of the HBV is increased, a higher oxygen tension gradient is required for oxygen release to the tissue. Thus oxygen release is shifted to the downstream vasculature (26), redistributing oxygen in favor of ischemic, hypoxic tissues (25). The advantage of left-shifting HBV oxygen affinity for this purpose was repeatedly demonstrated in our previous works (5, 6). Furthermore, the HBVs increased plasma viscosity, which has been made responsible for improved microcirculatory blood flow (5, 6, 8, 17, 18), both enhancing shear stress on the vascular lining. In our previous studies, a beneficial effect of the left-shifted HBVs on ischemic flap tissue has been shown for several secondary end points including tissue energy metabolism (5), apoptosis (17), proinflammatory cytokines, and leukocyte activation (18).

To weigh the biological performance of the biomaterial against its possible adverse effects, we performed pathohistomorphological examinations in the organs at risk, which is where HBVs are degraded and excreted, such as in the liver, spleen, and kidney (20). Although our test animals received large amounts of HBV, it was completely metabolized after six days, and no tissue alterations or damages were seen at this time point. These findings are in line with extended histomorphological and laboratory investigations performed by Sakai et al. (21, 22) in rats.

From our results, we conclude that left-shifted HBVs may ameliorate the survival and primary healing of critically ischemic wound margins, possibly by stimulating eNOS-mediated neovascularization.

GRANTS

This research was supported by Swiss National Foundation for Scientific Research Grants 32-065149.01 and 32-108408.05; by the Department of Clinical Research, University of Berne, Switzerland; by a Health and Labour Science Research grant from the Ministry of Health, Labour and Welfare, Japan; and by from the Japan Society for the Promotion of Science Grant-in-Aid for Scientific Research B1930164.

REFERENCES


