Liposome-Encapsulated Hemoglobin Accelerates Gastric Wound Healing in the Rat

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Background: Liposome-encapsulated hemoglobin, a nanometer-sized artificial O₂ carrier with a high O₂ affinity (h-LEH), may facilitate O₂ delivery to surgical wounds and thereby accelerate healing after gastrointestinal surgery.

Methods: Ten mL/kg of h-LEH (n = 25), empty liposome (n = 21) or homologous washed red blood cells (RBC, n = 22) was intravenously infused prior to the creation of a 10 mm incision and interrupted suture closure of the gastric wall in rats. After two and four days, the stomach was excised and the bursting pressure was determined by gradually inflating the stomach with air. This procedure was followed by histological examinations.

Results: The bursting pressure of the surgical wound was significantly higher two days after surgery in the h-LEH-treated rats in comparison to the control rats that received either empty liposome or RBC transfusion (P < 0.05). The three groups displayed similar bursting pressures four days after surgery. Histological examinations revealed less neutrophil infiltration, better granulation, and more macrophage infiltration in the h-LEH-treated rats after two days; however, these differences were no longer significant four days after surgery.

Conclusion: The results suggest that h-LEH, but not a homologous transfusion or empty liposome, may accelerate early wound healing after a gastric incision and anastomosis in a rat model. (200 words)

Key words: Gastrointestinal Surgery, Wound healing, Artificial oxygen carrier, Liposome-encapsulated hemoglobin, Bursting pressure

INTRODUCTION

Post-operative wound healing is important for accelerating patients’ recovery as well as for preventing anastomotic dehiscence, which may otherwise lead to serious complications. Wound ischemia and/or hypoxia [1], anemia [2] and malnutrition [3] have been considered to be the major factors responsible for impaired wound healing. Because wound ischemia is believed to be of the utmost importance, studies have been conducted to evaluate its mitigation, including ischemic preconditioning [4] and supercharging with microsurgical techniques [5]. Preconditioning remains controversial, and supercharging is not always possible because of the clinical situation and the anatomy involved in the disease [5]. Therefore, wound healing is most often supported by indirect measures, such as transfusion to correct anemia [2] and parenteral alimentation to improve nutritional status [3]. We have previously explored the possible utility of liposome-encapsulated hemoglobin (LEH) as an artificial oxygen (O₂) carrier [6, 7]. Its characteristics include a comparable O₂-carrying capacity to red blood cells (RBC), nanometer size, and adjustable O₂ affinity. Unlike the existing cell-free hemoglobin-based O₂ carriers [8], LEH is encapsulated by the liposome (similar to RBC structure) and is therefore protected from the adverse effects of nitric oxide scavenging [6, 7]. Because of its size (230 nm), much smaller than that of RBCs, LEH is thought to freely perfuse collateral vessels and capillaries and prevent ischemic damage in a rat brain ischemia model [9]. Moreover, LEH has been modified to have a higher oxygen affinity (h-LEH; P₅₀ = 10 mmHg) in order to transport more O₂ than RBCs under hypoxic conditions [10]. Based on these characteristics, we evaluated the hypothesis that h-LEH may supply O₂ to foster aerobic metabolism in the surgical wound and thereby accelerate its healing in a rodent model of gastric incision and anastomosis.

MATERIALS AND METHODS

Liposome-Encapsulated Hemoglobin (LEH)

The relevant characteristics of LEH (Terumo Co., Ltd., Tokyo, Japan) have been previously reported [6, 7]. Briefly, it is a liposome capsule measuring a mean of 230 nm in diameter and containing hemoglobin eluted from human RBCs outdated for transfusion. The liposome capsule is coated with polyethylene glycol (to reduce aggregation and capture by the reticuloendothelial system) in order to prolong the circulation
half-life to 13 hours in the rodent [6, 7, 9, 10] and to 70 hours in the primate [7]. Insoluble hexaphosphate is included as an allosteric effector for 2, 3-diphosphoglycerate to adjust the O2-affinity of LEH (TRM-645, \( P_{50,0} = 40 \) to 50 mmHg [6, 7, 9, 10]) and to increase the O2-affinity (\( P_{50,0} = 10 \) mmHg, h-LEH) higher than that of rodent RBCs (\( P_{50,0} = 30 \) mmHg) in the current study (Fig. 1A). LEH is suspended in saline at a hemoglobin concentration of 6 g/dL or 25% of volume (LEHcrit). LEH is precipitated between plasma and RBCs by centrifugation at 50,000 x g for 2 hours. A sibling rat donated homologous blood, which was collected in a syringe containing citrate-phosphate-dextrose solution. The blood was then centrifuged to separate RBCs, which were diluted with saline and washed at least 3 times to 25% hematocrit to serve as control solution containing a comparable amount of hemoglobin as h-LEH. Empty liposome was prepared in exactly the same manner as LEH, except that saline was encapsulated instead of hemoglobin in order to create particles without O2-carrying capacity.

**Oxygen Content Calculation**

Based on the O2 dissociation characteristics (Fig. 1A), the O2 contents of plasma, h-LEH and RBC were calculated as follows:

Plasma (O2 mL/dL): PO2 x 0.0031

h-LEH (O2 mL/dL): \( S_{\text{Hb,LEH}} \times \text{LEHcrit} \)/25 x 6 x 1.39

RBC (O2 mL/dL): \( S_{\text{Hb,RBC}} \times \text{hematocrit} / 3 \times 1.39 \) with hematocrit: volume of RBC (%), LEHcrit: volume of h-LEH (%), \( S_{\text{Hb,LEH}} \): saturation of h-LEH (%), \( S_{\text{Hb,RBC}} \): saturation of RBC (%), and PO2: partial pressure of O2 (mmHg).

The O2 content in the whole blood is the sum of all three components, and the O2 content in the plasma fraction includes the sum of the O2 content in the plasma and h-LEH.

**Animals**

All experiments were approved by the institutional review board of Tokai University School of Medicine. The rats received humane care as required. Male 6–8-week-old Sprague-Dawley rats (210–260 g, mean 242 g) were used. The rats were anesthetized and randomly assigned to one of three groups. They were maintained with 2% ethane and O2 and infused with 10 mL/kg of h-LEH (n = 25), empty liposome (n = 21), or transfusion of the homologous washed RBC to the same amount of hemoglobin (n = 22). The infusion was performed over 10 minutes at a slow speed (2.1 to 2.6 mL/10 minutes) to avoid acute volume load. The rats received a 10 mm incision in the gastric body, which was suture-closed using 4 inverted stitches of monofilament absorbable suture (Opepolix, Alfresa-Pharma, Inc, Osaka, Japan) by the same surgeons (Y0 and YK). A blood sample was obtained immediately before (Pre) and after surgery (Post). After the operation, the infusion line was removed, and the animals were placed back in cages with room air and access to food and water ad libitum until bursting pressure determination after surgery.

**Bursting Pressure**

Either two days (n = 47) or four days after surgery (n = 21), the rats were anesthetized and killed by pentobarbital overdose. To determine the bursting pressure in each animal, the stomach was excised en-bloc and inflated by air at a speed of 150 mL/min with the esophagus and duodenum closed. Bursting pressure was determined at the highest pressure achieved prior to leaking or rupture of the incision. The excised gastric wall was subjected to histological examination.

**Morphological Studies**

After determining the bursting pressure, the gastric wall was excised so that the wound could be observed perpendicular to the incision line. The specimens were fixed and stained with hematoxylin-eosin (H & E). Histopathological analyses determined the degrees of neutrophil infiltration, granulation, and presence of macrophages, which were categorized as none (scored 0), mild (scored 1), moderate (scored 2) or severe (scored 3) by two independent observers blinded to the study protocol.

**Immunohistochemical Studies**

Immunohistochemical staining was performed for human hemoglobin or h-LEH and hypoxia-induced-factor-1α (HIF-1α [11]) 24 hours after surgery in additional rats treated with h-LEH or RBC transfusion.

**Statistics**

Data are presented as mean ± SD. The bursting pressure and histological scores of rats were averaged for each group and compared among groups by Kruskal-Wallis analysis. A P value of less than 0.05 was considered to be significant.

**RESULTS**

**RBC and h-LEH Volume Changes**

The measured volumes of RBCs (hematocrit) (Fig. 1B) showed that the RBC-treated rats had the highest hematocrit throughout the postoperative period. Although the other rats showed a 2% reduction early after surgery, their hematocrit increased to an identical level by 4 days after surgery. Ten mL/kg of h-LEH administration yielded an LEHcrit of 4.6%, which then decreased to 1.4% within 2 days and to an undetectable level after 4 days (Fig. 1B). The O2 dissociation characteristics (Fig. 1A) showed assumption of the blood O2 content for whole blood and in the plasma fraction (plasma and h-LEH) immediately before (Pre) and after surgery (Post) as well as at 2 days (POD 2) and 4 days (POD 4) after surgery in animals receiving h-LEH (h-LEH), empty liposome (EL), or transfusion of the equivalent amount of hemoglobin (RBC). Although the whole blood O2 content was similar in rats receiving the RBC transfusion or h-LEH early after surgery, the early post-operative O2 content in the plasma fraction was higher only in the rats that received h-LEH. Due to the gradual disappearance of h-LEH from the circulation (Fig. 1B), the difference in plasma O2 content diminished after two days and then disappeared after four days (Fig. 1C).
Fig. 1 O₂ dissociation characteristics (Panel A) are shown for rodent RBCs and h-LEH. Volume changes in RBCs (hematocrit) and h-LEH (LEHcrit) among the groups of rats receiving RBC (RBC, solid line), h-LEH (h-LEH, masked line) or empty liposome (EL, dotted line) demonstrated that surgical blood loss caused an approximately 2% reduction in hematocrit, which was reinstated after 4 days (Panel B). The O₂ content calculated from the hematocrit and LEHcrit for whole blood (Panel C, upper panel) and for the plasma fraction (Panel C, lower panel) demonstrated that h-LEH administration compensated for RBC loss and maintained the whole blood O₂ content similar to that observed in animals receiving RBC transfusion until the RBC level was replenished 4 days later when h-LEH was removed from the circulation.

**Bursting Pressure**

The h-LEH-treated animals had a significantly higher average bursting pressure after two days (Fig. 2) in comparison to rats treated with empty liposome (EL) or homologous transfusion (RBC). While there were no LEH-treated animals with a bursting pressure under 30 mmHg after two days, 14.3% (2/14) of the empty liposome-treated animals and 33.3% (5/15) of the RBC-transfused rats had a bursting pressure under 30 mmHg. Four days after surgery, each group displayed an increase in bursting pressure and there was no longer any significant difference among the treatment groups. Nonetheless, all LEH-treated animals (7/7) had a bursting pressure over 50 mmHg, in contrast to only 57% of rats treated with empty liposome (4/7) or RBC transfusion (4/7).

**Morphological Changes**

H & E staining of the suture line 2 days after surgery (Fig. 3A) disclosed that LEH-treated animals showed decreased neutrophil infiltration (Fig. 4A), increased granulation (Fig. 4B), and increased macrophage numbers (Fig. 4C) in comparison to the other groups (Fig. 3B). These differences disappeared 4 days after the operation as inflammation subsided and tissue granulation increased in the other two groups (Fig. 3C). Immunohistochemical staining for human hemoglobin or h-LEH 24 hours after surgery (Fig. 5A) disclosed heavy staining around the suture line, suggesting the perfusion of h-LEH to the incision and anastomosis. A consecutive section stained for HIF-1α [11] showed a decreased expression of HIF-1α-positive cells in the anastomosis in h-LEH-treated rats 24 hours
Fig. 2 Bursting pressures observed in the groups of animals treated with h-LEH (h-LEH), empty liposome (EL) and RBC (RBC) at two days (POD 2) and four days after surgery (POD 4). Although there was a significant difference in the average bursting pressure among the groups (*) only in POD 2, there were no h-LEH-treated animals with a bursting pressure less than 30 mmHg after two days or less than 50 mmHg after 4 days.

Fig. 3 Histological observation at a low magnification (Panel A) two days after surgery showed suppressed infiltration of neutrophils in an h-LEH-treated rat (LEH) in comparison to animals receiving empty liposome (EL) or RBC transfusion (RBC) with severe and diffuse neutrophil infiltration. H & E staining at a higher magnification in POD 2 (Panel B) disclosed marked macrophage infiltration (arrows) and regeneration of fibroblasts (triangles) in an h-LEH-treated rat in comparison to the other rats receiving empty liposome or RBC. H & E staining in POD 4 (Panel C) no longer showed any morphological differences among the treatment groups.
after surgery in comparison to rats receiving RBC transfusion (Fig. 5B).

**DISCUSSION**

Bursting pressure in all of the h-LEH-treated rats was equal to or greater than 30 mmHg, and the average bursting pressure in this group was statistically higher in comparison to the other treatment groups two days after surgery. Although the statistical difference disappeared four days after surgery, all of the LEH-treated rats had a bursting pressure over 50 mmHg, while a number of animals in the other treatment groups failed to achieve the same degree of wound healing in 4 days. Urschell [1] reported that ligation of the left gastric artery resulted in decreased mechanical strength of the anastomosis in rats early after esophagus and stomach anastomosis. Nonetheless, the bursting pressure in the animals with gastric ischemia later increased to the level of non-ischemic animals. These findings were consistent with those seen in the current study. Although mechanical strength eventually became equivalent among the groups, postoperative wound healing is most important early after gastrointestinal surgery when anastomatic disruption may cause major complications that often lead to fatal consequences.

Histological observation identified improvements in the LEH-treated rats that were similar to those seen in the functional evaluation (elevated bursting pressure). There was less inflammation and more tissue granulation at two days after surgery, but the differences between the groups were no longer significant after 4 days. The parallel improvements in functional as well as morphological findings in the postoperative course support the hypothesis that the LEH treatment (but not treatment with RBC transfusion or with small particles without O2-carrying capability) accelerated wound healing. The early postoperative macrophage infiltration might have been induced by the presence of human hemoglobin in the wound, because macrophages were not observed in rats treated with empty liposome, which was a lipid capsule of the same makeup. Although it is unclear from the current study, the presence of human hemoglobin in the wound, which is xenogenic to a rat, would not provide any benefit.

Transfusion is a common therapeutic option in surgery to supplement blood loss, improve O2 delivery and accelerate postoperative recovery [2]. Although most artificial O2 carriers have been developed for this purpose, so far no single carrier has been accepted as an RBC substitute for clinical use [8]. LEH as a blood substitute has previously been reported by Nogami et al. [12], who demonstrated that LEH with a low O2 affinity may be capable of maintaining the metabolism in rats suffering from hemorrhagic shock without scavenging nitric oxide. In the current study, both RBC transusions and empty liposomes failed to improve anastomotic mechanical strength. Because hematocrit in all animals remained over 40% in the current study, it is conceivable that an additional RBC transfusion
might not add any benefit in terms of wound healing as suggested by Mandai [2], who demonstrated that hemoglobin over 10 g/dL was sufficient for wound healing. The current observation may suggest that the O₂-carrying capacity of whole blood does not correlate with wound healing. The possibility that the presence of nanoparticles improves microcirculation is unlikely because the treatment with empty liposomes failed to provide any advantage.

Immunohistochemical staining for human hemoglobin revealed the presence of h-LEH in and around the wound. Although the amount of O₂ contained in h-LEH is small, 10 mL/kg of h-LEH (hemoglobin 600 mg/kg) may be adequate to preserve aerobic metabolism as suggested by the suppression of HIF-1α expression in the wound 24 hours after surgery. Similar protective effects of LEH have been demonstrated in cerebral ischemia and reperfusion, another model of local perfusion derangement, in rats [9, 10] as well as in non-human primates [Kawaguchi et al., in preparation]. In these dose-response studies, the minimal amount of LEH needed to yield cerebral protection was as small as 0.4 mL/kg (hemoglobin 24 mg/kg). Since RBC transfusion failed to afford any protection in wound healing or in brain ischemia [9], the presence of a hemoglobin-containing nanoparticles in the plasma may be important. In the brain ischemia model, a 1/5 dose of h-LEH was reported to be as effective as the LEH with a low O₂ affinity [10]. Because these LEHs are identical except for the amount of encapsulated allosteric effector [6, 7], the difference in their efficacy may suggest the importance of O₂ delivery, but not the presence of hemoglobin in the plasma per se [9].

In conclusion, the current results support our hypothesis that h-LEH may improve the aerobic metabolism and accelerate wound healing. These functions are not performed by homologous transfusion or by nanoparticles without an O₂-carrying capacity. While the current results are compatible with our hypothesis, the mechanism(s) remain unclear and should therefore be elucidated in future studies.

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