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Liposome-encapsulated hemoglobin alleviates hearing loss after transient cochlear ischemia: An experimental study in the gerbil

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HIGHLIGHTS

- We tested if post-ischemic administration of LEH alleviates cochlea damage.
- Post-treatment with LEH alleviated hearing impairment.
- Post-treatment with LEH alleviated inner hair cell loss.

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ABSTRACT

The effects of liposome-encapsulated hemoglobin (LEH), an artificial oxygen carrier, were experimentally investigated in gerbils in the context of alleviation of hearing loss after transient cochlear ischemia. Animals were randomly assigned to receive 2 mL/kg of either LEH ($P_{50}O_2$ = 15 mmHg) or saline 1 h after the experimental induction of 15 min of ischemia. Sequential recordings of auditory brainstem response (ABR) showed that administration of LEH prevented hearing loss due to cochlear ischemia. The mean ABR threshold at 32 kHz on day 1 was 21 ± 7 dB in the LEH group (n = 6) and 45 ± 6 dB in the saline group (n = 6). Thereafter, hearing impairment gradually improved up to day 7 in both groups. The animals were then subjected to histological study, which revealed that there was more substantial loss of the inner hair cells, but not the outer hair cells, in the saline group as compared to the LEH group. These results suggest that LEH is an efficient agent with regard to protection against hearing loss and underlying hair cell damager due to ischemic insult.

1. Introduction

Idiopathic sudden sensorineural hearing loss (ISSHL) is an acute form of hearing loss of unknown etiology involving mainly those aged 40–60 years, and its incidence in Japan is estimated to be 10–20 cases per 100,000 people per year [1]. While various etiologies have been proposed, circulatory disturbance and viral infection are considered the most plausible causes of the disease [2,3]. In a recently published ISSHL clinical guideline, only 2 treatments—corticosteroid therapy and hyperbaric oxygen therapy (HBOT)—are recommended as initial therapeutic options [4]. At present, corticosteroid therapy is accepted and used worldwide, but HBOT is not popular as it is an expensive and time-consuming intervention.

Liposome-encapsulated hemoglobin (LEH), an artificial oxygen carrier, was originally developed by the Terumo Corporation (Tokyo, Japan) as a substitute for red blood cells (RBCs). Recently, the use of LEH has been examined not only for blood transfusion [5] but also for oxygen delivery treatment for ischemic disease [6]. We previously reported that pre-ischemic administration of LEH prevented inner ear damage more effectively than that of homologous RBCs in an experimental study in gerbils [7]. On the basis of this finding, we considered that administration of this agent might work as a substitute for HBOT, as both treatments supply oxygen to the hypoxic site of the lesion. At present, however, the protective effects of LEH when administered after an ischemic insult remain unclear. The purpose of this study was to investigate whether LEH can prevent or alleviate ischemic damage to the cochlea when administered 1 h after ischemia.

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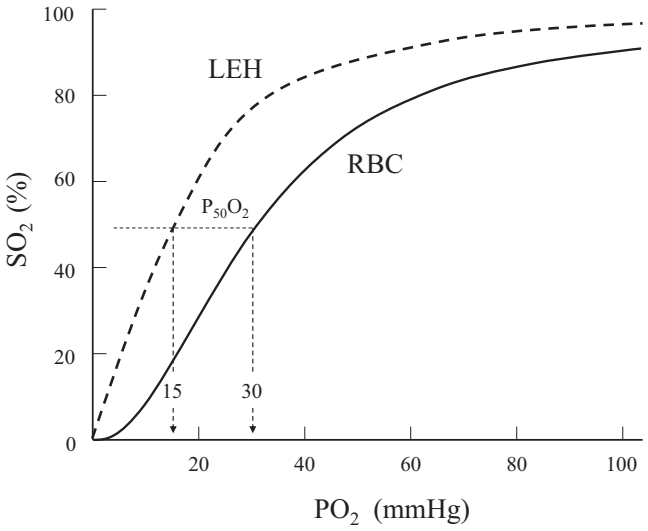

Fig. 1. Oxygen dissociation characteristics of LEH and RBC.

2. Materials and methods

The experiments were conducted in accordance with the Guidelines for Animal Experimentation at Ehime University Graduate School of Medicine. The animals received humane care as required by the institutional guidelines and the *Guide for the Care and Use of Laboratory Animals* [8].

2.1. LEH

The relevant characteristics of LEH have been reported elsewhere [9]. Briefly, the liposome capsule, measuring 230 nm in mean diameter, contains purified hemoglobin from outdated human RBCs. The liposome capsule is coated with polyethylene-glycol to reduce mutual aggregation, to avoid recognition by the reticuloendothelial cell system, and to prolong the half-life in circulation [10]. In its preparation, inositol-hexaphosphate was used to control O_2 affinity to $P_{50}O_2$ = 15 mmHg (Fig. 1). LEH was suspended in saline at a hemoglobin concentration of 6 g/dL or 20% by volume, and has reduced viscosity (2 cP) compared to blood (5 cP), with a specific gravity close to that of plasma. LEH resembles RBCs in structure, and in characteristics involving metabolic processes in the body, and it is stable in blood vessels. In rats the half-life of LEH is approximately 10 h [10].

2.2. Experimental animals

Adult male Mongolian gerbils (*Meriones unguiculatus*) weighing 60–80 g were purchased from Kyudo (Tosu, Japan) and used at 12–16 weeks of age. Anesthesia was induced with a mixture of 3% halothane and nitrous oxide:oxygen (7:3) gas, and maintained with a mixture of 1% halothane gas. The animals were ventilated artificially *via* a transoral tracheal tube (tidal volume 1 mL; respiration rate 70 min^{-1}). During the experiment, body temperature was monitored with a thermocouple probe (PTI-200, Unique Medical, Tokyo, Japan) placed in the rectum and kept at 37 ± 1 °C using a heating plate (HP-1M, Physitemp, Clifton, NJ, USA). The femoral vein was exposed to establish an intravenous infusion line by polyethylene catheter. The animals were randomly assigned to receive 2 mL/kg of LEH (n = 6) or saline (n = 6) 1 h after transient cochlear ischemia, induced over 10 min to avoid acute volume load, after which the catheter was removed and the vein ligated.

2.3. Transient cochlear ischemia and reperfusion

Following the procedures described by Hata et al. [11], transient cochlear ischemia was induced by temporarily occluding bilateral vertebral arteries in the neck, as gerbils lack posterior cerebral communicating arteries and the labyrinthine arteries are nourished solely by the vertebral-basilar system. The vertebral arteries were exposed bilaterally and dissected free from the surrounding tissues through a ventral midline incision in the neck with the animal in the supine position. Silk ligatures (4–0) were looped loosely around each artery. Ischemia was then induced in both cochleae by pulling the ligatures simultaneously using 5 g weights for 15 min. Subsequently, the threads were removed to allow reperfusion, which was confirmed by observation through an operating microscope. The wound was closed and animals were returned to cages with food and water available *ad libitum* until further testing.

2.4. Evaluation of hearing by auditory brainstem response (ABR)

Hearing was assessed prior to, and at 1, 4, and 7 days after ischemia. The animals were anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (1 mg/kg), and then ABR was recorded using a signal processor (NEC Synax 1200, NEC Medical Systems, Tokyo, Japan). Recording and reference needle electrodes were placed at the vertex and ipsilateral retroauricular area, respectively. As ischemic hearing loss below 4 kHz was minor in this animal model [12], we used pure tone bursts at 8 kHz, 16 kHz, and 32 kHz (rise and fall time 1 ms; duration 5 ms; repetition rate 15 s^{-1}) as auditory stimuli, delivered in an open field system (DPS-725, DIA Medical, Tokyo, Japan). The phases of the stimuli were alternated, thereby canceling interference of the cochlear microphonics. The speaker was located 20 cm in front of the left external auditory canal and masking noise (white noise) was administered to the right ear canal *via* a very small polypropylene tube. Responses to 1000 consecutive stimuli were averaged. The ABR threshold was determined by recording responses in 5 dB increments.

2.5. Histological study

The animals were sacrificed for histological study 7 days after recording their ABR. Under deep anesthesia, following removal of the otic bullae, the cochleae were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 into the scala tympani, and postfixed for 2 h with the same fixative at 4 °C. The specimens were immersed in phosphate-buffered saline (PBS) and the organ of Corti was dissected out using a surface preparation technique under an operating microscope. The walls of the bony cochleae were removed entirely without disrupting the organ of Corti. Then, the basal turn of the organ of Corti was isolated. Each specimen was stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA) diluted 250-fold in PBS containing 0.25% Triton X-100 and 1% bovine serum albumin for 30 min at room temperature. After rinsing in PBS, it was stained further with Hoechst 33342 (Calbiochem-Novabiochem, La Jolla, CA, USA) dissolved in PBS in a dark room for 1 h. It was again rinsed in PBS and mounted in carbonate-buffer glycerol (one part 0.5 M carbonate buffer at pH 9.5 to nine parts glycerol) containing 2.5% 1,4-diazabicyclo[2,2,2]octane to retard bleaching of the fluorescent signal. Fluorescence was detected using an Olympus BX60 microscope (Olympus, Tokyo, Japan) equipped with a green band pass (BP) 546, darb teiler spiegel (FT) 580, long pass (LP) 590 nm, and UV (BP 365, FT 395, LP 397 nm) filters. Rhodamine-phalloidin staining enables observation of the hair cell architecture, whereas Hoechst 33342 staining reveals that of the nuclei. The numbers of intact and dead hair cells at the basal turn were counted and the percentages of dead hair cells and intact hair cells were determined. Gerbils have