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● *Original Contribution*

EFFECTS OF LOW-INTENSITY FOCUSED ULTRASOUND ON THE MOUSE SUBMANDIBULAR GLAND

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Abstract—Ultrasound is expected to make a considerable contribution to drug delivery systems (DDSs). We tested the hypothesis that low-intensity focused ultrasound (LIFU) increases vessel permeability in the mouse submandibular gland without causing parenchymal damage. In a preliminary study, LIFU at 3 W/cm^2 with a 50% duty cycle for 2 minutes did not cause histologic damage. We therefore applied LIFU to mouse submandibular gland at these conditions before and after injecting horseradish peroxidase. Single labeling laser scanning confocal microscopy revealed positive horseradish peroxidase staining around the excretory ducts in the mucous-producing part of the gland, but absence of staining in control glands. Immunostaining for fibrinogen was positive in the same region. Fibrinogen is an intravascular protein that does not pass through intact vessels. These findings suggest that LIFU increases vessel permeability and disruption without destruction. It is anticipated that this process will be useful in establishing a DDS that uses LIFU. (E-mail: suzukim@med.oita-u.ac.jp)

Key Words: **Low-intensity focused ultrasound (LIFU), Vessel permeation, Drug delivery system (DDS), Mouse, Submandibular gland, Fibrinogen, Horseradish peroxidase (HRP).**

INTRODUCTION

Introduction and literature

Therapeutic ultrasound can affect biologic systems by two mechanisms, thermal and nonthermal. Nonthermal ultrasound energy has recently been applied for targeting and control of drug release (Harrison et al. 1991; Nelson et al. 2002). Such therapeutic ultrasound used in combination with drug delivery systems (DDSs) is expected to contribute considerably to various medical fields. For example, ultrasound energy can enhance the effects of thrombolytic agents such as urokinase and clinical trials of therapeutic ultrasound catheters for treatment of stroke are underway (Daffertshofer and Hennerici 2003; Francis et al. 1995; Tachibana 1992). Chemical activation of pharmacologic agents by ultrasound energy in the treatment of various cancers, including brain tumors, is also being studied (Mesiwala et al. 2002). Although ultrasound is known to cause transient permeability of cell membranes and small indentations or "pores" have been observed in cell membranes by means of electron microscopy *in vitro* (Tachibana et al. 1999), the mechanisms of sonoporation are not well understood. We hypothesized that low-intensity focused ultrasound (LIFU) increases vessel permeability in a targeted region of mouse submandibular gland without causing parenchymal damage. We tested this hypothesis in a target region of mouse submandibular gland as well as the histologic effectiveness of various intensities and durations of LIFU *in vivo*.

MATERIALS AND METHODS

The study was performed in accordance with the Law Concerning the Protection and Control of Animals (Law No. 105, 1 October 1973), Standards Relating to the Care and Custody of Laboratory Animals (Notification No. 9, 27 March 1980, Office of the Prime Minister) and Method for Sacrificing Laboratory Animals (Notification No.40, 4 July 1995, Office of Prime Minister). The animal-use protocol was approved by the Committee on Animal Experiments of Oita University (Approval No. E 028003, 11 June 2004).

Preparation of animals

Eighty 8-week-old male Sea:ddY mice were purchased from Seac Yoshitomi, Ltd. (Fukuoka, Japan) and

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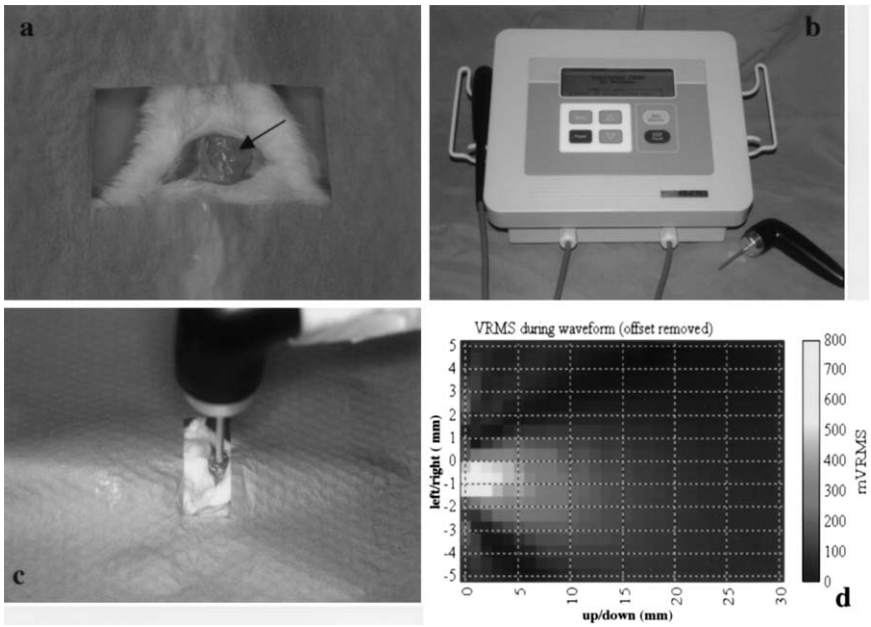

Fig. 1. (a) A bilateral submandibular incision was made to create an acoustic window for LIFU application. (b) Sonitron 2000 with probes (ultrasound device from Rich-mar Corp.). (c) Set-up for the *in vivo* experiments. The LIFU probe is mounted on the positioner and the tip of the probe gently touches the surface of the submandibular gland. Ultrasound transmission gel was used to couple the probe tip to the surface. (d) Characterization of the acoustic field. Peak positive pressure field generated by ultrasound at 1 MHz 3 W/cm^2 with a duty cycle of 100% with a 3-mm probe, as measured in water. The origin of the acoustic beam is at the left edge of the beam plot and its focal length was 3.5 to 4.0 mm from the tip of probe. The scale at the right of the beam plot gives LIFU power as a function of color: black = minimum; white = maximum.

were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). Adequate anesthesia was verified by absence of the corneal reflex and absence of limb withdrawal in response to noxious stimuli.

Experimental protocol

The submandibular gland membrane of each mouse was exposed by cervical skin incision (Fig. 1a) to ensure contact with the ultrasound probe. LIFU was applied through the membrane of the left submandibular gland with a single-element focused transducer and a 3 mm × 45 mm probe (Sonitron 2000, Rich-mar, OK) (Fig. 1b). The ultrasound frequency was fixed at 1 MHz; the intensity, duty cycle and duration varied. The surface of the submandibular gland was covered with sufficient sonic gel (Aquasonic 100, Parker Laboratories, Inc., NJ) to fill the gap between the membrane and LIFU probe (Fig. 1c). Characterization of the acoustic field of this system was shown in Figure 1d. The 3 W/cm^2 intensity ultrasound indicated 3 to 4 mm focal length from the tip of probe (close to the tip of the probe). At 3 W/cm^2, for instance, the ultrasound intensity was 3 W/cm^2 at the face of the probe and decreased linearly with depth.

Tissue collection

The mice were killed by deep anesthesia achieved by IP injection of pentobarbital. The anesthetized mice were perfused intracardially with physiological saline containing 0.1% heparin then by 10% neutral-buffered formalin. The right submandibular gland of each mouse was used for control, in addition to animals that were not operated on and did not receive ultrasound. Tissues were dehydrated sequentially through a graded series of alcohols, cleared in xylene and embedded in paraffin.

The effect of LIFU: Light microscopy

In preliminary studies, we developed the LIFU protocol that was used in the subsequent experiments of submandibular gland vessel disruption. LIFU was applied to the submandibular gland with a 3-mm probe under the following six experimental conditions: 3 W/cm^2 intensity, 100% duty cycle, at durations of 3 minutes, 2 minutes and 1 minute and 3 W/cm^2 intensity, 50% duty cycle, at durations of 3 minutes, 2 minutes and 1 minute. Nine mice were subjected to each of the six experimental conditions, and the mice were killed at 1 , 5, and 14 days after the ultrasound exposure (three mice at each time point for each of the six experimental conditions). To avoid thermal effects, the probe was