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Effect of therapeutic ultrasound on brain angiogenesis following intracerebral hemorrhage in rats

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ABSTRACT

Intracerebral hemorrhage (ICH) can produce severe neurological deficits in stroke survivors. However, few effective approaches are available to improve the recovery from ICH. Given that therapeutic ultrasound exposure can enhance on angiogenesis in peripheral tissues, the present study was designed to examine the effects of therapeutic ultrasound exposure on the brain angiogenesis following ICH. To this end, we applied once daily therapeutic ultrasound treatment to rats for 7 consecutive days after intracranial infusion of vehicle (Sham control) or collagenase (ICH). Repeated exposure to the low intensity of therapeutic ultrasound decreased behavioral scores in ICH rats, but not in sham control rats. Such an effect was correlated with an increased number of vessel-like structures and microvessels and PCNA positive cells in vWF-positive blood vessels in perihematomal brain tissues at post-ICH day 7. Furthermore, immunohistochemistry and western blotting results showed that ICH trigged the expression of extracellular matrix (ECM)-related molecules, including collagen Is, III, and IV, as well as integrins αvβ3 and α5β1, and exposure to therapeutic ultrasound increased the expression of these molecules. Therefore, our results indicated that repeated exposure to a low intensity of therapeutic ultrasound can increase the expression of collagen and integrins of ECM-related molecules, promote the formation of a large number of vessel-like structure and capillaries around the hematoma, and accelerate the recovery of neurological function impaired by ICH.

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Introduction

A common and often fatal subtype of stroke is intracerebral hemorrhage (ICH), which can produce severe neurological deficits in survivors. However, there is a lack of effective treatments that can improve the outcome in ICH patients at present, partly because of the few attentions on the development of effective approaches to improve the recovery from ICH.

The quality of recovery from ICH depends on the neovascularization, which can improve blood and oxygen supply in damaged brain tissue and help the brain repair the damage. Previous studies have demonstrated that several mechanisms, such as triggering of mechano-sensors, activation of endothelial cell growth, and sonoporation-activated alterations can contribute to the ultrasound-induced angiogenesis in peripheral tissue. Specifically, ultrasound exposure has been reported to produce shear stress on endothelial cells (Mizrahi et al., 2007). Thus, exposure to ultrasound may affect endothelial cell function *via* mechano-sensors embedded in endothelial cell membranes (Heo et al., 2014; Zaragoza et al., 2012). A recent study has also reported that exposure to therapeutic ultrasound can rescue necrotic limbs through augmenting microvascular growth and blood perfusion recovery after ischemic attack (Huang et al., 2014). Additionally, ultrasound exposure has been also shown to induce sonoporation on endothelial cell membrane leading to influx of calcium ion (Hassan et al., 2010). Such an increase of intracellular Ca^{2+} induced by ultrasound exposure may lead to increase of NO through the action of eNOS, which is a dominant modulator to enhance revascularization *in vivo* (Huang et al., 2014).

Additionally, it has been shown that the extracellular matrix (ECM) plays an important role in angiogenesis. Specifically, as the most abundant and most important insoluble fibrin in the ECM, collagen constitutes the skeleton of the cell to provide tensile strength and elasticity, plays a critical role in cell migration and development and in maintaining, repairing, and protecting the integrity of the organization (Myllyharju and Kivirikko, 2001). It has been reported that ECM, such as collagens I and III is closely related to the formation of new blood vessels (Majesky et al., 1991a). Besides collagens, integrins αvβ3 and α5β1 are also involved in the formation of new blood vessels. For instance, while integrins αvβ3 and α5β1 have a low level of expression on the

Abbreviations: ICH, intracerebral hemorrhage; ECM, extracellular matrix.

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surface of endothelial cells, tissue remodeling, such as tumors, trauma, and inflammation, can robustly enhance the expression levels of integrins αvβ3 and α5β1 (Boudreau and Varner, 2004).

However, little is known about the effects of therapeutic ultrasound exposure on brain angiogenesis following ICH. To this end, the present study was designed to examine the effects of therapeutic ultrasound exposure on brain angiogenesis following ICH. We hypothesized that repeated exposure to a low intensity of ultrasound could enhance the expression of ECM-related molecules, including collagens I, III, and IV, as well as integrins αvβ3 and α5β1, which might be correlated with the enhanced brain angiogenesis following ICH in rats.

Material and methods

Animals

Male Sprague–Dawley rats (n = 280) that weighed 275–300 g at the time of surgery were obtained from the Experimental Animal Science Center of Cangzhou Central Hospital, and were housed individually under 12 h light/dark cycle and had free access to food and water. All animal experiments were approved by the Institutional Animal Care and Use Committee of Cangzhou Central Hospital. The housing and treatment of the rats followed the guidelines of the "Guide for the Care and Use of Laboratory Rats" (Institute of Laboratory Animal Resources, Commission on Life Sciences 2011). All surgery was performed under isoflurane, and all efforts were made to minimize suffering.

Collagenase-induced intracerebral hemorrhage

The protocol of collagenase-induced ICH was modified based on an established method (Rosenberg et al., 1990). Briefly, animals were anesthetized with isoflurane (Abbott Laboratories, Abbott Park, IL, USA), and their head were placed into a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). The three dimensional coordinates of the head for each rat were recorded. The body temperature of the rats was monitored by a rectal thermometer and maintained at +37 °C using a CMA 450 temperature controller (Harvard Apparatus, Holliston, MA, USA). A longitudinal skin incision was made using scalpel to expose the skull and locate the bregma under aseptic conditions. A microinjection needle was then slowly, vertically inserted aiming at the globus pallidus (A/P −1.40 mm; M/L 3.20 mm; D/V 5.60 mm). A total volume of 2.5 μl vehicle (0.9% sterile saline; Sham control) or type VII collagenase (0.2 U/μl, Sigma Co., USA; ICH) was injected in 2 min. The needle was retained for 10 min after injection, and was then removed. The skin was sutured and disinfected subsequently.

Therapeutic ultrasound treatment

Animals started to receive therapeutic ultrasound treatment (once daily, 15 min) on the day after intracranial injections of either vehicle or collagenase for 7 consecutive days under isoflurane-induced anesthesia at the area above the collagenase injection site. Control rats did not receive ultrasound treatment but received isoflurane-induced anesthesia. Specifically, the focused ultrasound transducer was customized by an ultrasound electronic equipment factory (Lanhui ultrasound electronic equipment, Wuxi, China). The diameter of the transducer's membrane is 2.0 cm. This transducer had a 1.0 MHz center frequency, 75 mm focal depth, and 20 mm radius. The amplitude of pressure and the dimensions of ultrasound beam generated by this transducer were also measured by using a needle type hydrophone with a 0.2 mm diameter of needle (Spectris Instrumentation and Systems, Beijing, China) in a degassed water-tank. The lateral and axial full-width-at-half-maximum intensities of the ultrasound beam were 1.25 and 11.8 mm, respectively. According to previous literature, the skull of mice can attenuate the amplitude of the generated pressure by 18% (Choi et al., 2007). In our study, we have seen about 22% attenuation of amplitude in *ex vivo* rat skull experiment. This is likely due to the enhanced thickness of rat skull as compared with mice skull.

During the experiment, rat was anesthetized using 1.25–2.50% isoflurane and its head was mounted on the stereotaxic apparatus, and was adjusted to the position based on previously recorded three dimensional coordinates. The head hair was removed and an ultrasound coupling chamber was placed over the head of rat with a thin layer of ultrasound gel between the chamber and skull. The chamber was filled with degassed water. The transducer was mounted on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA), so that the position of the transducer could be adjusted three-dimensionally. The transducer was connected with a function generator (Crest Ultrasonics, Shanghai, China) and a 50-dB power amplifier (Crest Ultrasonics, Shanghai, China). The position of the transducer was calculated based on the position of the bregma of each rat, and corrected based on the dimensions of the transducer shape so that the transducer's beam axis was positioned aiming at the globus pallidus (A/P −1.40 mm; M/L 3.20 mm). The focus was placed 5.6 mm beneath the skull, overlapping with the collagenase injection site. Ultrasound was delivered to the hemorrhage area with an energy flux density of 0.2 W/cm^2 at a frequency of 1.0 MHz. The safety and the efficacy of such a low-frequency level of ultrasound wave to the organism have been examined in pilot experiments.

Behavioral observation

All the behavioral tests were conducted at day 7 after ICH following the last therapeutic ultrasound exposure. The Longa score was graded as the following: 0, no neurological deficit; 1, left front paw cannot fully extend; 2, walking in circle toward the contralateral side; 3, dumping toward the contralateral (paralyzed) side when walking; and 4, cannot autonomously walk with certain consciousness loss.

To evaluate the balance beam score, rats were placed 10 cm above the ground on an 80 × 2.5 × 2.5 cm stick to be allowed to walk, and the scores were graded as follows: 0, jump onto the balance beam and walk without falling; 1, can jump onto the balance beam and walk with less than 50% chances of falling; 2, can jump onto the balance beam and walk with more than 50% chance of falling; 3, can jump onto the balance beam with help from the ipsilateral hindlimbs but cannot move forward due to the paralyzed contralateral hindlimbs; 4, cannot walk on the balance beam but can sit on it; and 5, fall after being placed on the balance beam.

The Berderson score was calculated as follows. Rat tail was lightly grasped 10 cm above the desktop, and the score was graded as the following: 0, no neurological deficit; 1, flexion of the contralateral wrist and elbow and flexion adduction of the contralateral shoulder; 2, signs in score 1 plus reduced resistance when pushing toward the paralyzed side; and 3, circling toward the paralyzed side when acting (tail-chasing like).

Finally, we evaluated limb symmetry score. The rats were placed on a net with a mesh size of 2.3 × 2.3 cm, and the times of forepaws falling into the mesh were counted within 2 min. The formula was as follows: (number of wrong steps of the contralateral forepaws − number of wrong steps of the ipsilateral forepaws) / total number of steps. A positive number indicates functional deficit of the contralateral side, whereas a negative number indicates functional deficit in the ipsilateral side.

Immunohistochemistry

At day 7 after ICH, after anesthetization with an intraperitoneal injection of 10% chloral hydrate (400 mg/kg), rats were placed in supine position. A transverse incision was made under the xiphoid process, and the ribs were cut on both sides, to fully expose the heart. A syringe needle was inserted upward at a 45°–60° angle to the longitudinal axis between the trunk and the apex of the heart, into the ascending aorta. After fixing the needle with a hemostat and clamping

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