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Increasing of Blood-tumor Barrier Permeability through Paracellular Pathway by Low-frequency Ultrasound Irradiation In Vitro

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Abstract The research was conducted to study the increase of blood-tumor barrier (BTB) permeability through paracellular pathway by low-frequency ultrasound (LFU) irradiation in vitro. LFU (frequency=1.0 MHz) was performed to irradiate BTB model from the co-culture of rat C6 glioma cells and rat brain microvascular endothelial cells (RBMECs). The permeability of BTB was measured by transendothelial electrical resistance (TEER) and flux of horseradish peroxidase (HRP) assays after LFU irradiation. Western-blotting, immunohistochemistry, and immunofluorescence assays were used to investigate the changes of expressions and distributions of tight junction (TJ)-associated proteins ZO-1, occludin, and claudin-5. The TEER value began to decrease, and the minimum value appeared at 2 h, then gradually returned to the original level at 24 h after LFU irradiation. With time, flux of HRP gradually increased and reached the peak 2 h after LFU irradiation. The expressions of ZO-1, occludin, and claudin-5 in RBMECs decreased, and decreased most significantly at 2 h, then gradually restored to the original level at 24 h. Meanwhile, they were discontinuously distributed in the cellular boundaries after LFU irradiation. In summary, the expresstion of TJ-associated proteins was down-regulated, TJ was opened, and the permeability of BTB was increased through paracellular pathway by LFU irradiation.

Keywords Blood-tumor barrier · Low-frequency ultrasound · Tight junction · Tight junction-associated protein

Introduction

Because of the blood-tumor barrier (BTB), most of the available anticancer agents cannot be delivered into the brain tumor (Black and Ningaraj 2004). Some studies showed that, if the drug concentration increases by twofold in the tumor tissues, the efficacy for killing the tumor cells will increase tenfold (Samoto et al. 2001). In a word, the efficient delivery of drugs to tumor cells is crucial to effective chemotherapy of brain tumor.

It is not possible for therapeutic agents to exert pharmacological effects on brain unless they can go through blood–brain barrier (BBB), which consists mainly of endothelial cells with tight junction (TJ; Siegal and Zylber-Katz 2002). BTB is similar to BBB, which is located between tumor cells and microvessels formed by highly specialized endothelial cells. The permeability of BTB is higher than that of BBB (Yuan et al. 1994), but it is still a significant barrier for therapeutic drugs to penetrate (Erdlenbruch et al. 2003).

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There are two pathways of drugs being delivered into the brain tumor cells through the BTB (Anderson 2001). The one pathway is the paracellular (between cells) pathway, in which transported drugs go through endothelial TJ. The other pathway is the transcellular (through cells) pathway, in which transported drugs is taken up by the endothelial cell from the apical surface, transported across the cell, and released at the basolateral surface. Most of drugs are transported transcellularly depending on their physicochemical properties, and the paracellular pathway is usually the main way of absorption of hydrophilic drugs (Salama et al. 2006). BTB restricts the drugs being delivered into the brain tumor through paracellular pathway by complex endothelial TJ. TJ is the important structural and functional basis for keeping the integrality of BTB. TJs-associated proteins of ZO-1, occludin, and claudin-5 are mostly component of TJ, and the alteration of their structure plays a key role in regulating the function of TJ (Wolburg and Lippoldt 2002; Gloor et al. 2001). The permeability of BTB can be increased through paracellular pathway by TJ opening.

Some studies demonstrated that low-frequency ultrasound (LFU) irradiation can selectively open the BBB, and reversible increase the permeability of BBB (Hynynen et al. 2003; Gerriets et al. 2007; McDannold et al. 2007). Furthermore, other studies had shown that LFU can increase the permeability of BTB in vivo, without detectable neuron damage (Zhang et al. 2008). LFU irradiation provides new therapeutic strategies for delivery of anticancer agents through BTB into brain tumor. However, it is unclear that which pathway can increase BTB permeability by LFU irradiation.

In this study, we used the co-culture of rat C6 glioma cells and rat brain microvascular endothelial cells (RBMECs) to establish the BTB model in vitro, and the BTB model was irradiated by LFU of 1.0 MHz. It aimed to investigate the change of BTB permeability, and attempted to elucidate the increasing of BTB permeability through paracellular pathway by LFU irradiation.

Materials and Methods

Establishment of BTB Model In Vitro

Rat C6 glioma cells (obtained from the Cell Biology Department of the China Medical University) were maintained in complete culture media (high glucose DMEM, 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin).

Neonatal Wistar rats were used for the isolation of primary BMECs. Animals (<1 week) were sacrificed by CO_2 inhalation. Immediately after death, craniotomy was performed, and the entire cerebrum was dissected free of meninges. The protocol to establish BMECs followed what was described in previous studies (Scott and Bicknell 1993; Biegel and Pachter 1994; Biegel et al. 1995). All cells were grown as monolayers in tissue culture flasks in a humidified atmosphere (5% CO_2/95% air) at 37°C.

BTB models were established in vitro as described by Hurst and Fritz (Hurst and Fritz 1996). First, rat C6 glioma cells were trypsinized, then the cells were plated onto the underside of the collagen-coated polytetrafluoroethylene polyester polystyrene Transwell insert (0.4-μm pore size; Corning Costar) with suitable culture medium containing 10^6 cells. The Transwell was inverted after 2 h and fresh medium added. When 80% of C6 glioma cells were confluent, RBMECs were seeded on the upper chamber of the Transwell insert (approximately 10^5 cells). The cells became confluent after 7–10 days of coculture. From then onward, the medium was renewed every 2 days. The cells were used to following experiments after 7–10 days of coculture.

LFU Irradiation

An ultrasound beam of 1 MHz generated by a pulse-waved transducer of EMS-9 ultrasound apparatus (Shenzhen Delica Company, China) was used to irradiate vertically upward from the bottom of culture plate. Coupling agent was used between probe and culture plate. The parameters of the transducer were set as following: repetition frequency of 400 Hz, exposure time of 15 s, diameter of 2 cm, focusing distance of 5 mm, acoustic power of 10 mW, peak negative pressure amplitude of 0.44 MPa, spatial peak temporal intensity of 0.14 W/cm^2.

Experimental Groups

There were seven groups in our study (n=8 per group). It was divided into control group and experimental groups. The control group was not treated by LFU irradiation, while the experimental groups were irradiated by LFU. According to the time after LFU irradiation, experimental groups were divided into: 0.5-, 1-, 2-, 4-, 8-, and 24-h groups.

MTT (3-[4.5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide; Thiazolyl Blue) Assay for Cell Viability

MTT assay was used to detect RBMECs cell viability after LFU treatment. At first, we plated 200 μl of cell suspension of RBMECs in each well of a 96-well flat-bottom microtiter plate. Cells were incubated for 5 days at 37°C in a humidified 5% CO_2 atmosphere. The control group was not treated by LFU irradiation, while the experimental group was irradiated by LFU. We added 20 μl of MTT solution prepared at a concentration of 5 mg/ml in phosphate-buffered saline (PBS) to each well and continued the cell culture for another 4 h at 37°C. Then, the MTT solution was discarded and the 150 μl dimethyl sulfoxide

(DMSO) was added to each well. The colorless DMSO turned purple, and was read immediately at 570 nm in a scanning multi-well spectrophotometer (SpectraMax M5, Molecular Devices corporation, USA).

Measurement of Transendothelial Electrical Resistance

Transendothelial electrical resistance (TEER) was determined with a Millipore electrical resistance system (Millicell-ERS; Millipore, Bedford, MA). To avoid fluctuations in temperature, TEER was measured at 37°C with the aid of a heating plate. The final TEER values were calculated as Ω·square centimeter by multiplying by the surface area of the Transwell insert.

Measurement of the Horseradish Peroxidase Flux

The BMECs monolayers in Transwell inserts were cocultured with C6 glioma cells and transferred to a new 12-well plate. Horseradish peroxidase (HRP; Sigma-Aldrich) at 0.5 μmol/L in serum-free DMEM was added to the upper compartment of the Transwell system. After LFU irradiation for the indicated time, the media from the lower chamber were collected, and the HRP content of the samples was assayed colorimetrically. The HRP flux was expressed as picomoles passed per square centimeter surface area.

Western Blot Assay

Western blot analysis was performed to investigate the expression of ZO-1, occludin, and claudin-5 in the BMECs of experimental and control groups. To obtain the cell membrane fraction, the BMECs of each group were homogenized in 1 ml lysis buffer A (2 mM EDTA, 10 mM EGTA, 0.4%NaF, 20 mM Tris–HCl, protease inhibitors, pH 7.5) at 4°C. Samples were centrifuged 14,000×*g* for 30 min, and the supernatant was transferred to a separate tube. After that, 150 μl buffer A with 1% Triton X-100 at 4°C was added to the pellet. The pellet was disrupted with an ultrasonic crusher, and the samples were placed on a rocker at 4°C for 1 h. Samples were then centrifuged at 14,000×*g* for 30 min at 4°C as described above. The supernatant ("membrane fraction") was collected and the proteins concentration determined by the Coomassie G250 binding method. Equal amounts of proteins (10–20 μg) were separated by SDSPAGE and processed for immunoblotting with antibodies for ZO-1 (diluted 1:200; Zymed), occludin (diluted 1:300; Santa Cruz Biotechnology), claudin-5 (diluted 1:300; Santa Cruz Biotechnology) and a polyclonal antibody β-actin (diluted 1:300; Santa Cruz Biotechnology) was applied as an inner control. Immune complexes were visualized by enhanced chemiluminesecence (ECL kit; Santa Cruz Biotechnology).

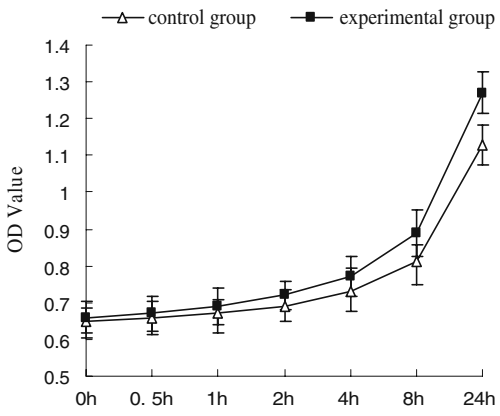

Fig. 1 MTT assay revealed the effect of LFU treatment on BMECs cell viability. The OD_{570} value of control group and experimental group are shown. The RBMECs cell survival curve of experimental group are similar to that of control group. These are not statistically significant in both control group and experimental group. Values represent means±SD; $n=8$, each; *white up-pointing triangle* is control group; *black square* is experimental group

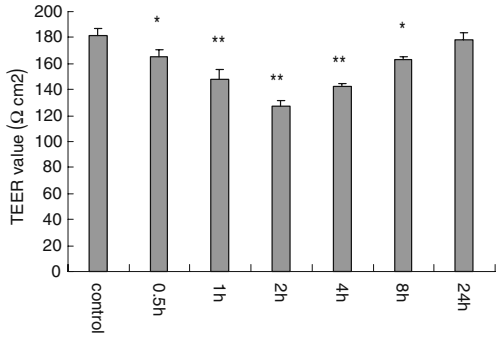

Fig. 2 Effect of LFU irradiation on TEER. The change of BMECs TEER value of control group and experimental groups are shown. Values present means±SD; $n=8$, each; *single asterisk,* $P<0.05$; *double asterisk,* $P<0.01$ vs. control group

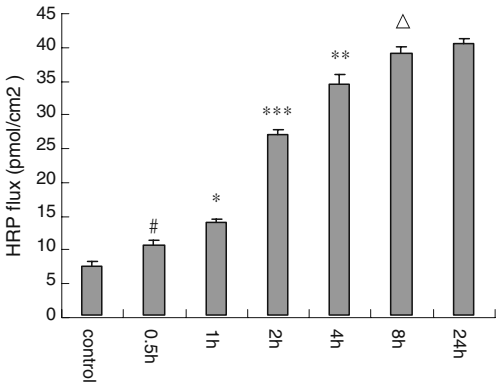

Fig. 3 Time-dependent changes of HRP flux by LFU irradiation. The change of BMECs HRP flux of control group and experimental groups are shown. Values present means±SD; $n=8$, each; *number sign*, $P<0.05$ vs. control group; *single asterisk,* $P<0.05$ vs. 0.5-h group; *triple asterisk,* $P<0.001$ vs. 1-h group; *double asterisks* $P<0.01$ vs. 2-h group; *white up-pointing triangle,* $P<0.05$ vs. 4-h group

All the protein bands were scanned using ChemiImager 5500 V2.03 software, and the integrated density values (IDV) were calculated by computerized image analysis system (Fluor Chen 2.0) and normalized with that of β-actin.

Immunohistochemistry and Immunofluorescence Assays

Immunohistochemistry and immunofluorescence were used to detect expression and localization of ZO-1, occludin, and claudin-5 in BMECs. The BMECs of experimental and control groups were taken then fixed with 4% paraformaldehyde solution and permeabilized with 0.5% Triton X-100. Immunohistochemistry stain was carried out in accordance with S-P method, and incubated with mouse polyclonal anti-ZO-1 antibody (diluted 1:50; Zymed), rabbit polyclonal antioccludin antibody (diluted 1:150; Santa Cruz Biotechnology), and goat polyclonal anticlaudin-5 antibody (diluted1:150; Santa Cruz Biotechnology) at 4°C overnight. Negative controls were conducted by exchange of primary antibody for PBS (0.01 mol/L), colored with DAB, counterstained with hematoxylin, then mounted with neutral balsam, and then coverslipped. Photographs were taken with a Nikon digital camera on an Olympus microscope. Indirect immunofluorescence was used to incubate with anti-goat IgG conjugated with FITC (diluted 1:100, Sigma–Aldrich) or anti-rabbit IgG conjugated with TRITC (diluted 1:100, Sigma–Aldrich) for 1 h at 37°C in darkness, and mounted by buffering glycerol. Images were captured using an Olympus BX 60 upright fluorescence microscope with appropriate filters and objectives. For semiquantitatively measuring the immunoreactivity of ZO-1, occludin, and claudin-5 in BMECs, the images were analyzed using a computer-aided image analying system (Image-Pro Plus 6.0).

Statistics Analysis

All results are expressed as the mean±SD for each group. A Student's *t* test was performed to determine the significant difference between two groups. One-way ANOVA and post hoc comparisons (Bonferroni test) were utilized to determine the significant differences among multiple groups. $P<0.05$ was considered statistically significant.

Results and Discussion

The Effect of BMECs Cell Viability by LFU Irradiation

The present study revealed that the permeability of BBB can be increased at some extent of acoustic intensity by

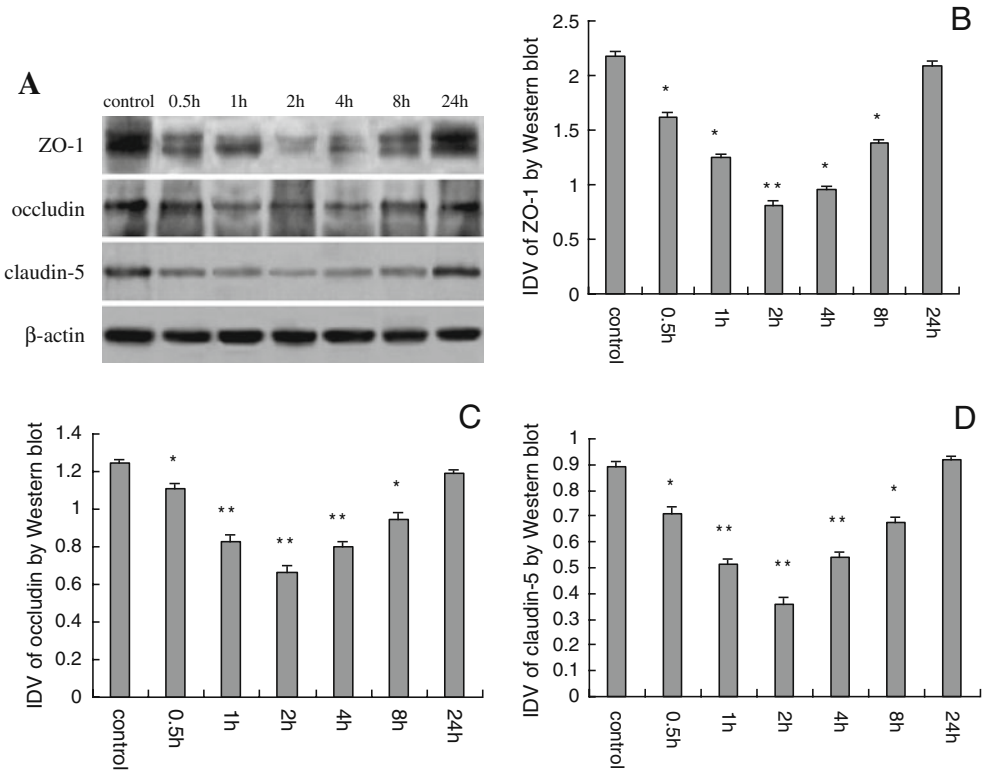

Fig. 4 Western blot analysis revealed LFU irradiation decreasing of protein level of ZO-1, occludin, and claudin-5 in BMECs of BTB in vitro (**a**). From *left to right*, the *lanes* are control group, 0.5-, 1-, 2-, 4-, 8-, and 24-h groups respectively. IDV of ZO-1 (**b**), occludin (**c**), and claudin-5 (**d**) are shown. Values represent means±SD; $n=8$, each; *single asterisk*, $P<0.05$; *double asterisk*, $P<0.01$ vs. control group

ultrasound irradiation without necrosis of the brain tissue (Hynynen et al. 2005). In this study, the purpose of MTT assay was to investigate the effect of LFU irradiation on RBMECs cell viability. As shown in Fig. 1, RBMECs of experimental group were growth in normal after LFU irradiation, the RBMECs cell survival curve of experimental group were similar to that of control group. These differences were not statistically significant in both control group and experimental group. The results of MTT assay suggested that LFU irradiation in the condition of our experimental parameters could not induce necrosis of RBMECs. It was feasible that we adopted LFU irradiation at this acoustic intensity to investigate the change of BTB permeability by LFU irradiation in vitro.

The Change of BTB Permeability by LFU Irradiation

Recent research showed that the permeability of BTB can be increased by LFU irradiation (McDannold et al. 2008; Bing et al. 2009). The LFU (frequency range in 20 kHz~1 MHz) has characteristics of penetrating into the skull easily, and the sonic energy absorbed little damage to the normal brain tissues (Daffertshofer et al. 2005; Yin and Hynynen 2005). Another study had suggested that LFU irradiation combined with bradykinin in small dose increase the permeability of BTB in vivo (Zhang et al. 2009).

In this study, we used LFU to irradiate BTB model in vitro. The experiments using TEER and HRP flux assays were performed mainly to evaluate BTB permeability. As shown in Fig. 2, TEER significantly decreased compared with the control group from 0.5- to 8-h group by LFU irradiation, and the minimum of TEER appeared at 2 h, then gradually increased and restored to the original level at 24 h after LFU irradiation. Meanwhile, we observed that LFU irradiation induced a significantly increase of HRP flux in a time-dependent manner. From the difference in two adjacent groups, we could deduce that the peak of HRP flux appeared at 2 h after LFU irradiation (Fig. 3). These results suggested that there was an increase of the BTB permeability by LFU irradiation in vitro.

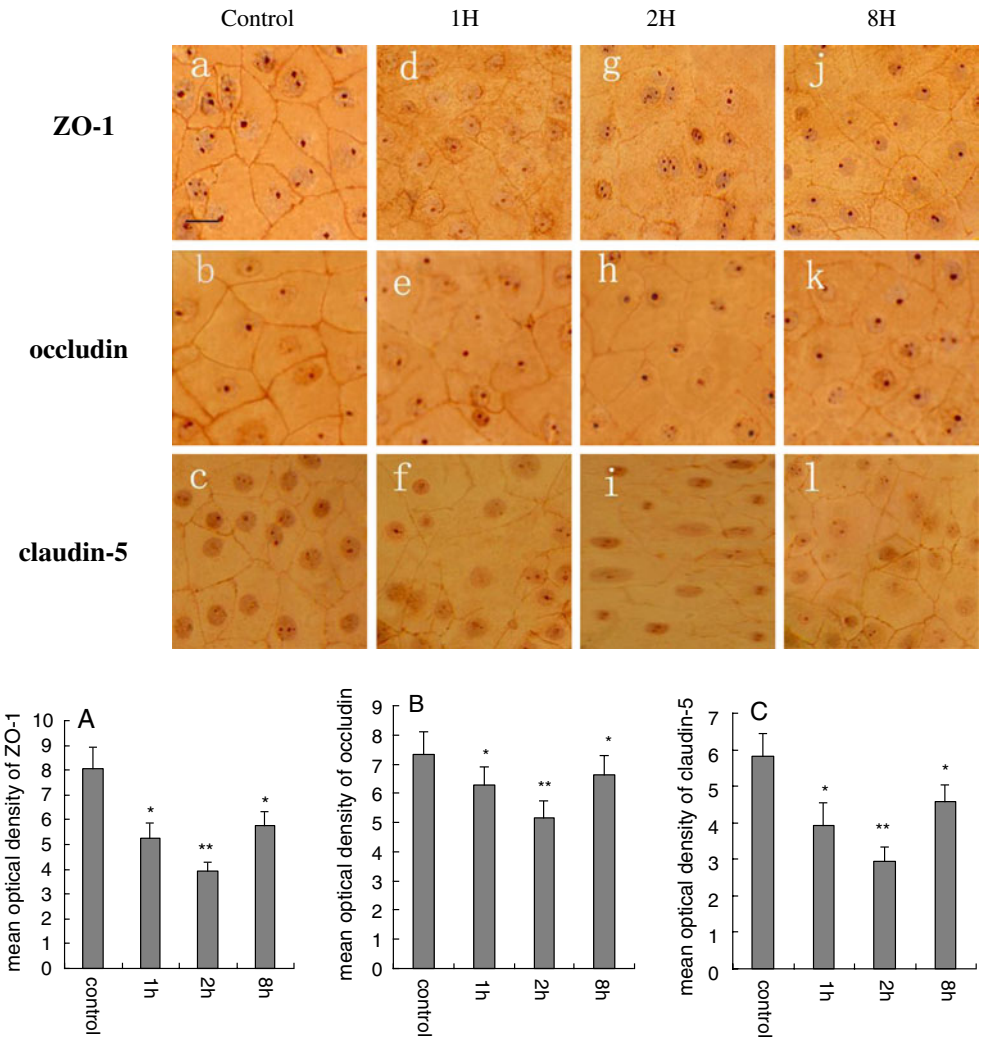

Fig. 5 Effect of LFU irradiation on expression and distribution of occludin, claudin-5, and ZO-1 in RBMECs were analyzed by immunohistochemistry (S-P method). RBMECs of control group and experimental groups (1-, 2-, and 8-h groups) are shown. ZO-1, occludin, and claudin-5 were localized to the cell–cell boundaries with continuous distribution in BMECs of control group (**a**–**c**). After LFU irradiation, ZO-1, occludin, and claudin-5 were distributed discontinuously in the boundaries of RBMECs at 1-h group (**d**–**f**) and 8-h group (**j**–**l**), and even were absented in the cellular boundaries at 2-h group (**g**–**i**). The immunoreactivity of ZO-1, occludin, and claudin-5 in BMECs of experimental groups was attenuated. Mean optical densities of ZO-1 (**a**), occludin (**b**), and claudin-5 (**c**) are shown. Values are means±SD; $n=8$, each; *single asterisk*, $P<0.05$; *double asterisk*, $P<0.01$ vs. control group. *Scale bars*= 20 μm

TEER is determined by TJ of adjacent BMECs, and the integrity of TJ was mainly responsible for the brain endothelial permeability (Harhaj and Antonetti 2004). TEER is an ideal permeability marker to determine whether TJ is open, and TEER is an important indicator to BTB permeability through paracellular pathway. Some scholars used HRP as a tracer to study the mechanism of opening of BBB by LFU irradiation, indicating that the opening of BBB can be performed through transcellular pathway or paraceller pathway (Hynynen et al. 2005; Sheikov et al. 2008). Other studies demonstrated that BTB could be opened through transcellular pathway by LFU irradiation in vivo (Xia et al. 2009). In this study, TEER was decreased in the first, and then gradually restored to original; the change of TEER was reversible after LFU irradiation. This result indicated that TJ of BMECs had caused a process of closing to opening and closing again by LFU irradiation, and BTB permeability in vitro was increased by TJ opening.

The Change of Expressions and Distributions of TJ-associated Proteins ZO-1, Occludin, and Claudin-5 by LFU Irradiation

According to the results of BTB permeability mentioned above, LFU irradiation could open the TJ, but the precise molecular mechanism of LFU irradiation opening TJ is unclear. To make the mechanism of LFU irradiation opening of the TJ clear, in this study, the expressions and distributions of TJ-associated proteins ZO-1, occludin, and claudin-5 were measured with Western blot and immunohistochemistry assays in the BTB vitro model.

Western blot analysis showed that the protein expression levels of ZO-1, occludin, and claudin-5 in BMECs cytomembrane significantly decreased compared with the control group from 0.5- to 8 h group by LFU irradiation, and the lowest level appeared at 2 h, then gradually returned to the original level at 24 h after LFU irradiation

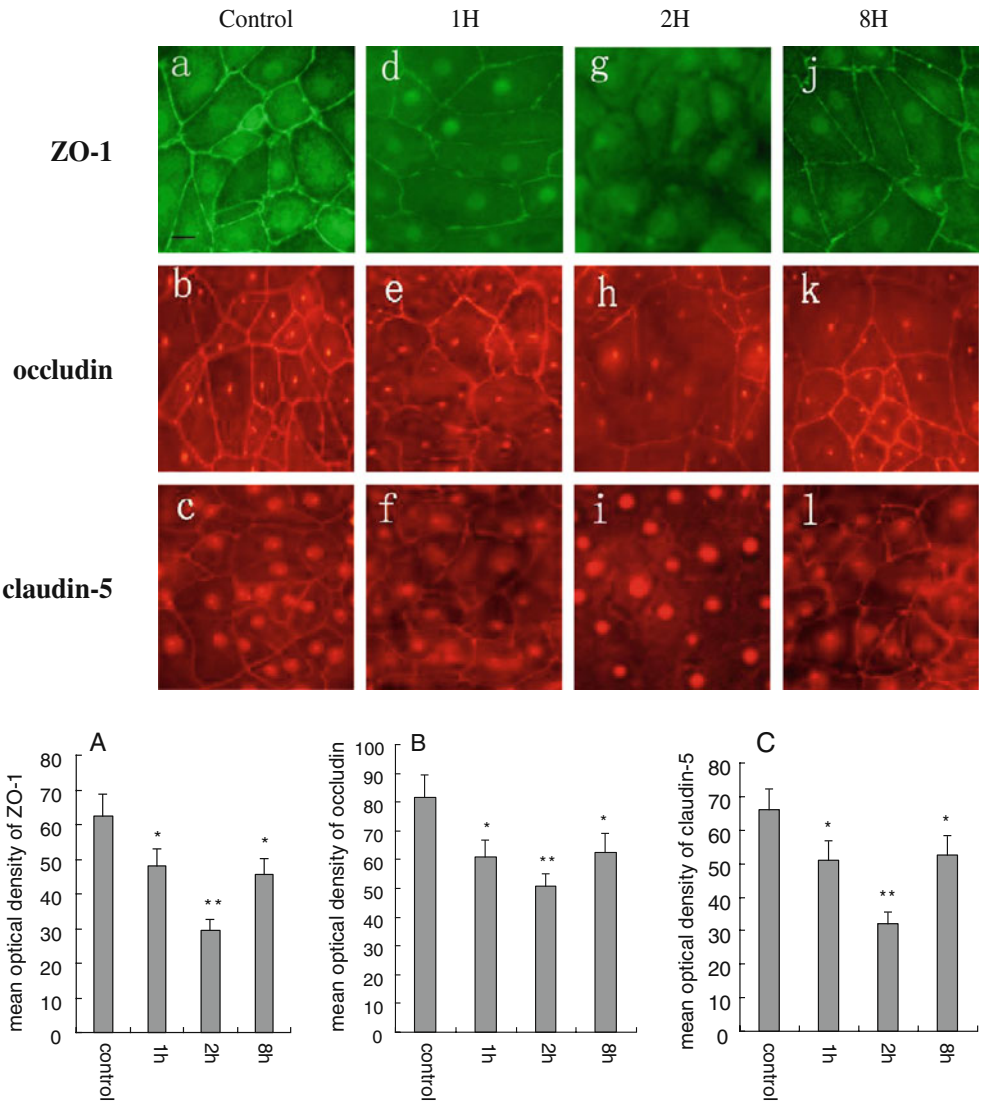

Fig. 6 Immunofluoresence localization of expression of ZO-1, occludin, and claudin-5 in RBMECs of control group and experimental groups (1-, 2- and 8-h group) are shown. The results of immunofluoresence were similar to those of immunohistochemistry. ZO-1, occludin, and claudin-5 were localized to the cell–cell boundaries with continuous distribution in BMECs of control group (**a**–**c**). After LFU irradiation, ZO-1, occludin, and claudin-5 were distributed discontinuously in the boundaries of RBMECs at 1-h group (**d**–**f**) and 8-h group (**j**–**l**), and even were absented in the cellular boundaries at 2-h group (**g**–**i**). The immunoreactivity of ZO-1, occludin, and claudin-5 in BMECs of experimental groups was attenuated. Mean optical densities of ZO-1 (**a**), occludin (**b**), and claudin-5 (**c**) are shown. Values are means ±SD; $n=8$, each; *single asterisk*, $P<0.05$; *double asterisk*, $P<0.01$ vs. control group. *Scale bars*=20 μm

(Fig. 4). There were down-regulations of protein expression levels of ZO-1, occludin, and claudin-5 in BMECs cytomembrane by LFU irradiation in vitro.

The results of immunohistochemistry and immunofluorescence analysis revealed that ZO-1, occludin, and claudin-5 were localized to the cell–cell boundaries with continuous distribution in BMECs of control group. After LFU irradiation, the immunoreactivity of ZO-1, occludin, and claudin-5 in BMECs of experimental groups were attenuated, and they were discontinuously distributed, even absented in the cellular boundaries. The decreasing of ZO-1 and claudin-5 were more obvious than that of occludin at 2 h after LFU irradiation (Figs. 5 and 6). The results of immunofluoresence were similar to those of immunohistochemistry, and the results of immunohistochemistry and immunoflurescence analysis was coincident with that of Western blot analysis. These data suggested that TJ disassembly in BMECs, which were co-cultured with C6 glioma cells, was induced by LFU irradiation.

It is the role of TJ opening that increase the permeability of BTB through paracellular pathway. Some studies have shown that TJ localizes among cells of brain capillary endothelium, which is an important structural and functional basis of maintaining the BBB integrity. TJ is a static structure, and it can be rapidly disassembled and reorganized in response to various extracellular stimuli (Gumbiner 1996; Nusrat et al. 2000). TJ is made up of transmembrane proteins of occludin and claudins, peripheral membrane protein family of zonula occludens (ZOs), adhesion molecules, etc. (Wolburg and Lippoldt 2002; Gloor et al. 2001). ZO-1, occludin, and claudin-5 are representative of TJ-associated proteins, which play important roles in regulating the function of TJ. ZO-1 is the first identified protein of TJ proteins. It is a member of the MAGUK protein family (Stevenson et al. 1986), and increasing evidence indicates that the loss of ZO-1 could result in a disorganization of TJ (Youakim and Ahdieh 1999). Occludin is the best-known integral membrane protein of TJ, which is believed to be directly involved in its barrier and fence functions (Hirase et al. 1997). Claudin-5 is a major cell adhesion molecule of tight junctions in brain endothelial cells, and it may be directly involved in the establishment of the BBB and regulation of the BBB permeability (Nitta et al. 2003). Our results showed that LFU irradiation reduced the protein expression and changed the distribution of ZO-1, occludin, and claudin-5, and then the most obvious change appeared at 2 h after LFU irradiation (Figs. 3, 4, and 5). The time point at which change of ZO-1, occludin, and claudin-5 reached their peak were the same as that at which TEER and HRP flux reach their peak. The change of expression levels of TJ-associated proteins was coincident with that of BTB permeability. These results indicated that LFU irradiation could affect the tightness of TJ by down-regulating the expression of ZO-1, occludin, and claudin-5. The decreasing of TJ-associated proteins resulted in opening TJ, and BTB permeability increased through paracellular pathway.

In short, with BTB models in vitro, we prove that LFU irradiation opens TJ and increases the permeability of BTB by down-regulation of expression level and altering distribution of TJ-association proteins ZO-1, occludin, and claudin-5. By combining the recent research with our study, we conclude that LFU irradiation opens BTB and increases the permeability by paracellular pathways. However, some mechanisms will still be investigated further, for example, how LFU irradiation decreases TJ-associated proteins through signal regulation-dependent pathways.

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References

Anderson JM (2001) Molecular structure of tight junctions and their role in epithelial transport. News Physiol Sci 16:126–130

Biegel D, Pachter JS (1994) Growth of brain microvessel endothelial cells on collagen gels: applications to the study of blood–brain barrier physiology and CNS inflammation. In Vitro Cell Dev Biol 30A:581–588

Biegel D, Spencer DD, Pachter JS (1995) Isolation and culture of human brain microvessel endothelial cells for the study of blood–brain barrier properties in vitro. Brain Res 692:183–189

Bing KF, Howles GP, Qi Y, Palmeri ML, Nightingale KR (2009) Blood-brain barrier (BBB) disruption using a diagnostic ultrasound scanner and definity in mice. Ultrasound Med Biol 35:1298–1308

Black KL, Ningaraj NS (2004) Modulation of brain tumor capillaries for enhanceed drug delivery selectively to brain tumor. Cancer Control 11:165–173

Daffertshofer M, Gass A, Ringleb P, Sitzer M, Sliwka U, Els T, Sedlaczek O, Koroshetz WJ, Hennerici MG (2005) Transcranial low-frequency ultrasound-mediated thrombolysis in brain ischemia: increased risk of hemorrhage with combined ultrasound and tissue plasminogen activator: results of a phase II clinical trial. Stroke 36:1441–1446

Erdlenbruch B, Schinkhof C, Kugler W, Heinemann DE, Herms J, Eibl H, Lakomek M (2003) Intracarotid administration of short-chain alkylglycerols for increased delivery of methotrexate to the rat brain. Br J Pharmacol 139:685–694

Gerriets T, Walberer M, Nedlmann M, Bachmann G, Kaps M (2007) Blood–brain barrier disruption by low-frequency ultrasound. Stroke 38:251

Gloor SM, Wachtel M, Bolliger MF, Ishihara H, Landmann R, Frei K (2001) Molecular and cellular permeability control at the blood–brain barrier. Brain Res Brain Res Rev 36(2):8–264

Gumbiner BM (1996) Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell 84:345–357

Harhaj NS, Antonetti DA (2004) Regulation of tight junctions and loss of barrier function in pathophysiology. Int J Biochem Cell Biol 36:1206–1237

Hirase T, Staddon JM, Saitou M, Ando-Akatsuka Y, Itoh M, Furuse M, Fujimoto K, Tsukita S, Rubin LL (1997) Occludin as a possible determinant of tight junction permeability in endothelial cells. J Cell Sci 110(Pt 14):1603–1613

Hurst RD, Fritz IB (1996) Properties of an immortalised vascular endothelial/glioma cell co-culutre model of the blood-brain barrier. J Cell Physiol 167:81–88

Hynynen K, McDannold N, Vykhodtseva N, Jolesz FA (2003) Non-invasive opening of BBB by focused ultrasound. Acta Neurochir Suppl 86:555–558

Hynynen K, McDannold N, Sheikov NA, Jolesz FA, Vykhodtseva N (2005) Local and reversible blood-brain barrier disruption by noninvasive focused ultrasound at frequencies suitable for trans-skull sonications. Neuroimage 24:12–20

McDannold N, Vykhodtseva N, Hynynen K (2007) Use of ultrasound pulses combined with definity for targeted blood-brain barrier disruption:a feasibility study. Ultrasound Med Biol 33:584–590

McDannold N, Vykhodtseva N, Hynynen K (2008) Effects of acoustic parameters and ultrasound contrast agent dose on focused–ultrasound induced blood-brain barrier disruption. Ultrasound Med Biol 34:930–937

Nitta T, Hata M, Gotoh S, Seo Y, Sasaki H, Hashimoto N, Furuse M, Tsukita S (2003) Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. J Cell Biol 161:653–660

Nusrat A, Turner JR, Madara JL (2000) Molecular physiology and pathophysiology of tight junctions IV. Regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells. Am J Physiol Gastrointest Liver Physiol 279: G851–G857

Salama NN, Eddington ND, Fasano A (2006) Tight junction modulation and its relationship to drug delivery. Adv Drug Deliv Rev 58:15–28

Samoto K, Perng GC, Ehtesham M, Liu Y, Wechsler SL, Nesburn AB, Black KL, Yu JS (2001) A herpes simplex virus type 1 mutant deleted for gamma 34.5 and LAT kills glioma cells in vitro and is inhibited for in vivo reactivation. Cancer Gene Ther 8:269–277

Scott PA, Bicknell R (1993) The isolation and culture of microvascular endothelium. J Cell Sci 105:269–273

Sheikov N, McDannold N, Sharma S, Hynnen K (2008) Effect of focused ultrasound applied with an ultrasound contrast agent on the tight junctional integrity of the brain microvascular endothelium. Ultrasound Med Biol 34:1093–1104

Siegal T, Zylber-Katz E (2002) Strategies for increasing drug delivery to the brain: focus on brain lymphoma. Clin Pharmacokinet 41:171–186

Stevenson BR, Siliciano JD, Mooseker MS, Goodenough DA (1986) Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. J Cell Biol 103:755–766

Wolburg H, Lippoldt A (2002) Tight junctions of the blood–brain barrier: development, composition and regulation. Vasc Pharmacol 38:323–337

Xia CY, Zhang Z, Xue YX, Wang P, Liu YH (2009) Mechanisms of the increase in the permeability of the blood-tumor barrier obtained by combining low-frequency ultrasound irradiation with small-dose bradykinin. J Neuro Oncol 94:41–50

Yin X, Hynynen K (2005) A numerical study of transcranical focused ultrasound beam propagation low frequency. Phys Med Biol 50:1821–1826

Youakim A, Ahdieh M (1999) Interferon-γ decreases barrier function in T84 cells by reducing ZO-1 levels and disrupting apical actin. American Physiological Society 276(5Pt 1):G1279–G1288

Yuan F, Salehi HA, Boucher Y, Vasthare US, Tuma RF, Jain RK (1994) Vascular permeability and microcirculation of gliomas and mammary carcinomas transplanted in rat and mouse cranial windows. Cancer Res 54:4564–4568

Zhang Z, Xia CY, Liu YH, Xue YX (2008) Selective permeation of the blood-tumor barrier in rats by low frequency ultrasound treatment. Chinese J Ultrasound Med 24:581–584

Zhang Z, Xia C, Xue Y, Liu Y (2009) Synergistic effect of low-frequency ultrasound and low-dose bradykinin on increasing permeability of the blooa-tumor barrier by openjing tight junction. J Neurosience Res 87:2282–2289