Original Article

Altered Expression of Matrix Metalloproteinases and Tight Junction Proteins in Rats following PEMF-Induced BBB Permeability Change*

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Abstract

Objective To investigate the expression of occludin, ZO-1, MMP-2, and MMP-9 in cerebral microvasculature following Pulse Electromagnetic Field (PEMF) induced BBB permeability change.

Methods Sprague-Dawley rats were randomized into PEMF and sham exposed groups (*n*=8). After exposure to PEMF at 0.5, 1, 3, 6, and 12 h, BBB permeability was measured by Evans-Blue extravasation. The expression of occludin, ZO-1, MMP-2, and MMP-9 were detected by real-time quantitative reverse transcriptase PCR and western blotting. MMP-2 and MMP-9 activity were detected by EnzChek gelatinase assay.

Results Compared with the sham group, PEMF exposure led to increased permeability of the BBB to EB, which was prolonged after exposure. BBB permeability became progressively more severe, and recovered at 6 h. The gene and protein expression of occludin and ZO-1 were significantly decreased, while MMP-2 and MMP-9 expression were significantly increased after exposure to PEMF. All levels of expression recovered 12 h following PEMF.

Conclusion Changes to BBB permeability were related to the alteration expression of tight junction proteins and matrix metalloproteinase after exposure to PEMF.

Key words: Pulse electromagnetic field; Blood-brain barrier; Cerebral microvasculature

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INTRODUCTION

The blood-brain barrier (BBB) is a vascular system that regulates the passage of materials between the peripheral circulation and the central nervous system (CNS). The brain microvascular endothelium is distinguished by the presence of the BBB, consisting of epithelial-like tight junctions (TJs), that restrict paracellular permeability, highly regulated membrane transporters, that mediate the passage of molecules through the endothelium, and metabolic enzymes[1-2]. Recent studies found that Streptozotocin-induced diabetes mellitus led to increased BBB permeability. This change was associated with specific alterations in TJ proteins. Increased matrix metalloproteinase (MMPs) activity may play a role in the increase in permeability of the BBB[3]. Occludin is a transmembrane protein that functions at the TJs as both a structural and a signaling protein which restricts permeability to low molecular mass molecules and increases electrical resistance of barrier tissues, which is critically important for the barrier function of the BBB[4-5]. ZO-1 is the primary cytoplasmic accessory TJ protein,

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which links transmembrane proteins of the TJs to the actin cytoskeleton. This interaction is likely to be critical for the stability and function of the TJs. Dissociation of ZO-1 from the junctional complex is often associated with increased BBB permeability[6-7]. Experimental evidence indicates that MMP-mediated TJ disruption is an important mechanism leading to an excessive increase in BBB permeability. Occludin and ZO-1 are vulnerable to attack by MMPs[8]. MMPs are zinc-dependent endopeptidases that specialize in the degradation of components of the extracellular matrix and basement membrane of the cell, and are implicated in many brain disorders associated with BBB leakage[9]. MMP-2 and MMP-9 are formed *via* complex interactions of cytoskeletal proteins amongst the TJ proteins, and digest the endothelial basal lamina, which is essential for maintaining BBB integrity. MMPS are therefore mediators of BBB compromize[10].

BBB permeability has been shown to increase in connection to mobile phone use and exposure to an electromagnetic field (EMF)[11-13]. Pulsed Electromagnetic Field (PEMF) stimulation is a local, safe, and noninvasive therapeutic method. Recent studies suggest that BBB permeability alters after exposure to PEMF[14], which might be useful for local delivery of a drug or gene to a specific region in the brain through transient opening of the BBB. The goal of the present study was to investigate alteration in the expression of occludin, ZO-1, MMP-2, and MMP-9 in cerebral microvasculature, with BBB permeability change after exposure to PEMF.

MATERIALS AND METHODS

Animals and PEMF Exposure

Male Sprague-Dawley rats weighing 200-220 g were purchased from the Experimental Animal Center, Fourth Military Medical University (Xi'an, China). Rats were randomly assigned to PEMF (0.5, 1, 3, 6, and 12 h) and sham groups (n=8 in each group). Rats in the PEMF groups were whole-body exposed to PEMF (200 pulses of 200 kV/m at 1 Hz). The PEMF generator was developed and tested in the Northwest Institute of Nuclear Technology (Xi'an, China), and was designed to deliver pulses with 3.5 ns rising time, 14 ns pulse width, and amplitude up to 200 kV at 1 Hz repetitive rate. These parameters are based on Semiconductor Opening Switch technology.

Measurement of BBB Permeability

BBB permeability was measured using the leakage of Evans Blue (EB) dye as previously described[15]. Rats were injected with 4 mL/kg 2% EB (Sigma, St. Louis, MO) which was allowed to circulate for 30 min. After rats were anesthetized, brain tissue was harvested, weighed, homogenized, and sonicated in 50% trichloroacetic acid (TCA), and centrifuged at 10 000 rpm for 5 min. The supernatant was retained and read on a spectrophotometer at 610 nm wavelength against a concentration curve. EB leakage was expressed as μg/g brain tissue.

Isolation of Cerebral Microvessels

Cerebral microvessels were isolated from freshly removed brains following a previously described protocol, with some minor modifications[16]. After rats were anesthetized, the brain was removed from the skull, and the brainstem and cerebellum dissected away from the cerebral hemispheres. The meninges and choroid plexuses were then removed on ice. The hemispheres were homogenized in fivefold volume microvessel isolation buffer, pH7.4 (138 mmol/L NaCl, 4 mmol/L KCl, 2.2 mmol/L $CaCl_2$, 10 mmol/L HEPES), suspended in an equal volume 20% BSA, and centrifuged at 5 800 g for 10 min at 4 °C. The pellets were resuspended in 500 μL 20% bovine serum albumin (BSA) and centrifuged again. The final pellets were passed through a 100 μm mesh. The filtrates were centrifuged at 600 g for 10 min at 4 °C, and the resulting pellets were resuspended in 0.25 mL buffer.

Western Blot Analysis

Cerebral microvessels were homogenized at 4 °C in RIPA buffer (Beyotime Biotechnology, Suzhou, China), and sonicated for 10 s. After centrifugation at 12 000 g for 5 min, proteins were extracted, and then quantified using the bicinchoninic acid method with BSA as standard. Proteins were electrophoresed in SDS-polyacrylamide gels and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, USA) using standard procedures. Membranes were blocked at 4 °C for 1 h in Tris buffer saline containing 0.05% Tween 20 (TBS-T) and 5 wt% non-fat dry milk, then incubated overnight at 4 °C with rabbit polyclonal antibodies for occludin, and ZO-1 (Zymed, Inc. South San Francisco, CA, 1: 500); rabbit polyclonal antibodies against MMP-2 and MMP-9, and mouse monoclonal antibody directed towards β-actin (Santa Cruz Biotechnology, CA, 1:200). Membranes were washed several times with TBS-T, and incubated with appropriate HRP-conjugated secondary antibody (Santa Cruz Biotechnology, USA,

CA, 1: 5 000) for 30 min at room temperature. After further extensive washing, the membranes were developed with the ECL detection system (ECLplus, Millipore, Billerica, CA). Band intensity was measured by densitometry using Quantity-One software (Bio-Rad Lab, Hercules, CA). Results are expressed as percentage of the housekeeping protein and β-actin, to normalize for loading variations.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from each cerebral microvessel sample using TRIZOL reagent (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's protocol. Synthesis of cDNA and quantitative real-time PCR reactions were performed using PrimeScript TMRT reagent Kit (Takara, DaLian, China). Primers were designed using Primer 3.0 software. The forward and reverse primer sequences were as follows: 5'-CCCAGGAGATTCCTCT GACCTT-3' and 5'-CTTCGTGGGAGCCCTTTTAGA-3' (occludin, 101 bp); 5'-TGTGAGTCCTT CAGCTGTGGA-3' and 5'-GGAACTCAACACA CCACCATT-3' (ZO-1, 103 bp); 5'-CCCCGATGCTGATACTGA-3' and 5'-CTGTCCGCCAAATAAACC-3' (MMP-2, 152 bp); 5'-AAATGTGGGTGTACACAGGC-3' and 5'-TTCACCCGGT TGTGGAAACT-3' (MMP-9, 310 bp); 5'-CACCCGCGA GTACAACCTT C-3' and 5'-CCCATACCCACCATCACAC C-3' (β-actin, 207 bp). The β-actin gene was used as an internal standard. The Delta-delta Ct (DDCt) method was used to transform Ct values into relative quantities with standard deviation. Changes were expressed as a percentage of the controls.

MMPs Activity Assays

Total gelatinase activity was measured in homogenates of cerebral microvessels by fluorometry using an EnzChek gelatinase assay kit (Invitrogen Corporation, Carlsbad, CA). The assay was performed according to the manufacturer's recommendations, and the fluorescent intensity was measured in duplicates with a spectrofluorometer. Data were expressed as MMP activity in mU collagenase per mg total protein.

Statistical Analysis

Data are expressed as $\bar{x} \pm s$. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Newman-Keuls post hoc test. P<0.05 was considered statistically significant.

RESULTS

Evans Blue Extravasation

The permeability of brain tissue was assessed using EB extravasation analysis, where the permeability marker was EB (Figure 1). Compared with the control group, EB leakage increased to 2.67±0.07 at 0.5 h (P<0.05), reached a peak of 3.25±0.08 at 3 h (P<0.01), then declined and recovered to control levels at 6 h after exposure to PEMF. These results suggested that exposure to PEMF induced an increase in BBB permeability.

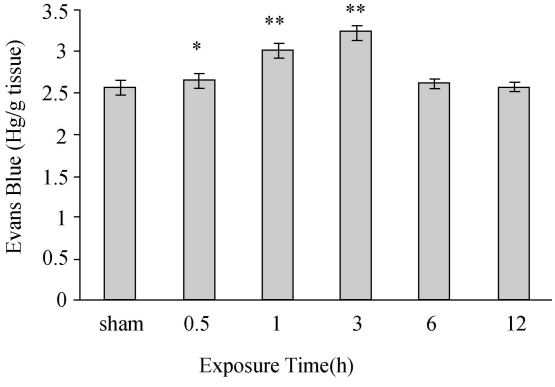

Figure 1. Evans blue extravasation in brain regions after exposure to PEMF. ***Note.*** Data presented as $\bar{x} \pm s$, n=8 in each group. $^{*}P$<0.05 and $^{**}P$<0.01 *versus* sham.

Effect of PEMF on TJ Expression

To determine if increased BBB permeability was due to an alteration of cerebral microvascular TJs, the mRNA and protein expression of TJ-associated proteins in cerebral microvessels, occludin and ZO-1, was assessed using real-time PCR and western blot analyses. As shown in Figure 2, mRNA and protein expression levels of occludin were significantly decreased to 35.85±7.74 and 40.61±6.17, respectively, at 1 h (P<0.01) after exposure to PEMF. mRNA and protein expression then increased to 74.25±4.54 and 72.89±4.35, respectively, at 3 h post PEMF exposure (P<0.01) and recovered to control levels at 6 h after exposure to PEMF (Figure 2B). The mRNA and protein expression levels of ZO-1 were significantly decreased to 67.34±8.47 and 76.28±7.31, respectively, at 1 h (P<0.01) following PEMF exposure and then peaked at 54.71±3.90 and 66.61±6.43, respectively, at 3 h (P<0.01) post exposure, but returned to control levels at 12 h after exposure to PEMF (Figure 2C).

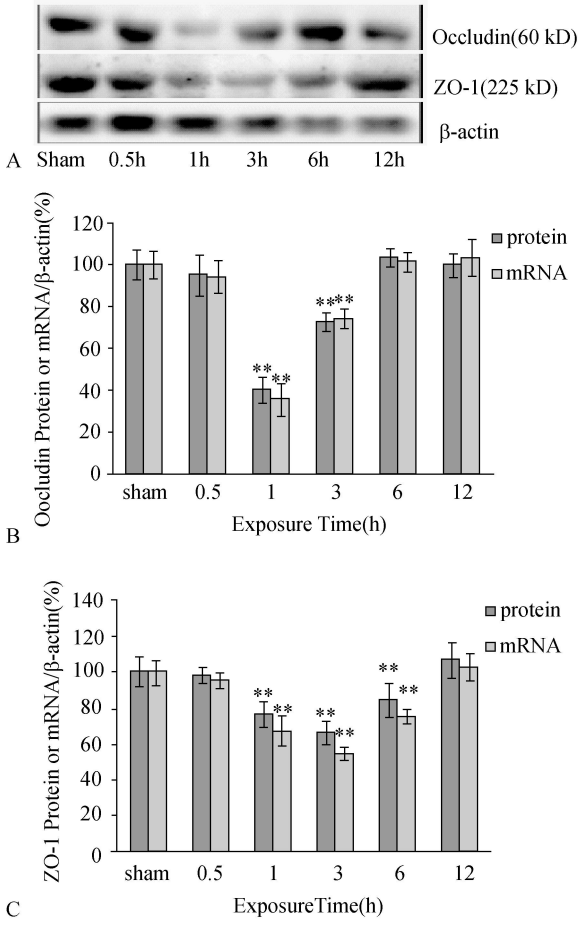

Figure 2. The mRNA and protein expression levels of occludin and ZO-1 in cerebral microvessels. **A**. A representative western blot of occludin, ZO-1 and β-actin. **B.** The expression levels of occludin in cerebral microvessels. **C.** The expression levels of ZO-1 in cerebral microvessels. *Note.* Changes were expressed as a percentage of the controls. Data are presented as $\bar{x} \pm s$, n=8 in each group. $^{**}P$<0.01 versus sham.

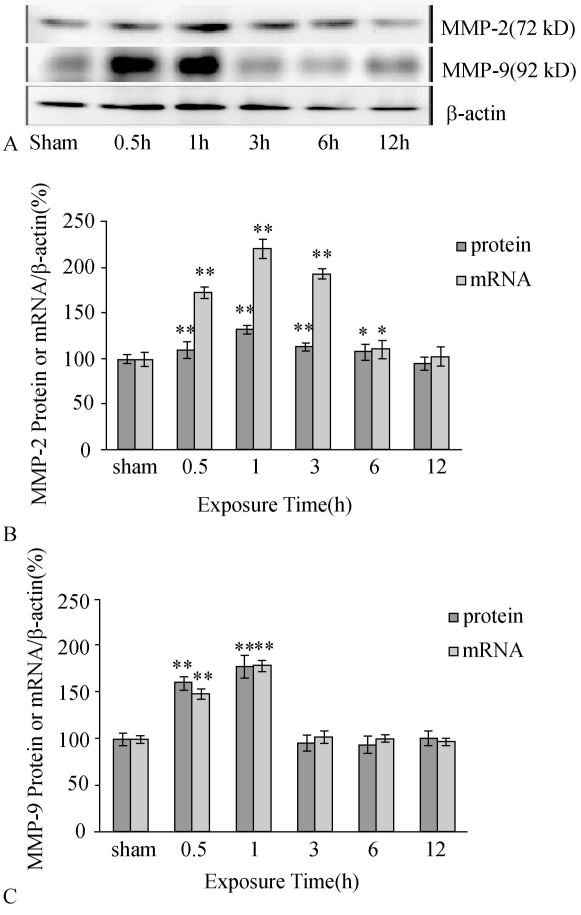

Figure 3. mRNA and protein expression levels of MMP-2 and MMP-9 in cerebral microvessels. **A**. Representative western blot of MMP-2, MMP-9 and β-actin. **B**. The expression levels of MMP-2 in cerebral microvessels. **C**. The expression levels of MMP-9 in cerebral microvessels. *Note.* Changes are expressed as a percentage of the controls. Data are presented as $\bar{x} \pm s$, n=8 in each group. $^{*}P$<0.05 and $^{**}P$<0.01 *vs.* Sham.

Effect of PEMF on MMP-2/9 Expression

Figure 3 shows the alteration in MMP expression. The mRNA and protein expression levels of MMP-2 were significantly increased to 172.74±6.341 and 109.64±9.05, respectively, at 0.5 h (P<0.01), peaked at 220.81±10.22 and 132.49±5.03 at 1 h (P<0.01), declined at 3h and recovered to control levels at 12 h after exposure to PEMF (Figure 3B). The mRNA and protein expression levels of MMP-9 were significantly increased to 149.26±5.5 and 161.29±7.53, respectively, at 0.5 h (P<0.01), peaked at 180.34±6.16 and 179.64±12.25 at 1 h (P<0.01), fell and returned to control levels at 3 h after exposure to PEMF (Figure 3C).

Effect of PEMF on Total Gelatinase Activity

As shown in Figure 4, total gelatinase activity in cerebral microvessels of PEMF and sham groups was determined. Compared with control, a significant up-regulation in cerebral microvessel gelatinase concentration was observed at 0.5 h (P<0.01), peaking at 1 h (P<0.01), declining at 3 h and returning to control levels at 12 h after exposure to PEMF.

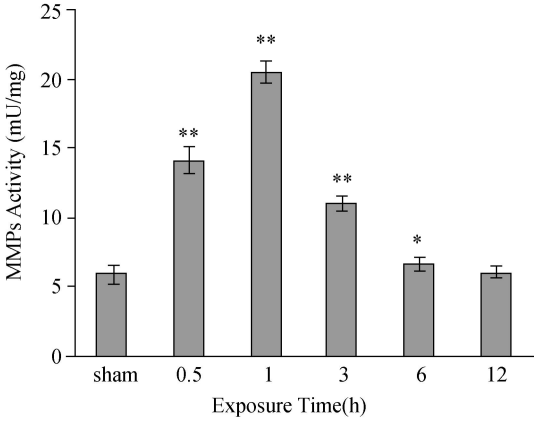

Figure 4. MMPs activity in cerebral microvessels after exposure to PEMF. *Note.* Data were presented as $\bar{x} \pm s$, n=8 in each group. *P<0.05 and **P<0.01 *versus* sham.

DISCUSSION

PEMF induces electric fields in the body that can potentially alter biological functions. Previous studies found a significant increase in homogenized brain content of EB after 4 weeks of 3h per day exposure to 50 Hz electromagnetic field[18]. In this study, we investigated BBB permeability after PEMF exposure using EB. The results indicate that PEMF exposure under our conditions, 200 kV/m, 1 Hz, 200 pulses, could transiently alter the permeability of BBB in rats.

We observed that the reduction of occludin and ZO-1 expression was closely correlated with the degree of enhancement of EB leakage. BBB permeability to EB increased concurrently with decreased production of TJs. As BBB permeability increases, ZO-1 appears to be less tightly associated with β-actin, perhaps indicating a disruption between the TJ scaffold and the cytoskeleton. Occludin plays a dynamic, functional role in regulating TJ integrity. Numerous phosphorylation sites allow occludin to rapidly respond to environmental stimuli[19]. Our data demonstrated that reductions in occludin expression may decrease paracellular permeability, resulting in an increased flux between BBB endothelial cells. In a previous study, we observed that occludin expression was not significantly changed in the frontal cortex compared with the control[20]. It seems that the importance of occludin in mediating epithelial barrier function appears to be tissue dependent.

MMP-2 and MMP-9 are large molecules, which restrict their proteolysis mainly to the immediate pericellular environment[21]. The total MMP activity in cerebral microvessels seems to be associated with the level of expression of MMP-2 and MMP-9. Matrix-degrading proteases are important in many normal and pathological processes. The activation reaction proceeds on the membrane surface if proteolysis is spatially constrained. MMP-2 is constitutively expressed, making it available for early tissue injury. MMP-9 is a proinflammatory protease that is released during the neuroinflammatory response by the stimulation of cytokines and immediate early genes. We clearly demonstrate that the elevated expression of MMP-2 and MMP-9 coincided with the loss of expression of occludin and ZO-1 in cerebral microvessels after exposure to PEMF. Results indicated that there is an important mechanism underlying the observed BBB permeability increase after PEMF exposure. The degradation of occludin and ZO-1 by MMPs may disrupt the stability of the TJ complex. These studies suggested that the increased BBB permeability was not due to changes in vascular volume or increased vascular trapping, most accounted for by increased paracellular diffusion between brain microvascular ECs. Occludin and ZO-1 expression was not altered at 0.5 h, although BBB permeability increased. ZO-1 and MMP-2 expression was altered at 6 h, although BBB permeability declined and recovered. These differing studies reflect the complexity of the formation and maintenance of TJs and may be a result of diverse signaling pathways.

In conclusion, BBB permeability was selectively increased after exposure to PEMF, which was related to the expression levels of occludin and ZO-1 which were selectively decreased, whilst MMP-2 and MMP-9 levels were increased. BBB disruption is sometimes accompanied by red blood cell extravasations, indicating a mild vascular injury. Further studies are necessary to investigate brain injury and address the safety of this method for opening the BBB, and appraising the safety of this technique for future clinical applications.

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