# Detrimental Effect of Electromagnetic Pulse Exposure on Permeability of *In Vitro* Blood-brain-barrier Model<sup>\*</sup>

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## Abstract

**Objective** To study the effect of electromagnetic pulse (EMP) exposure on permeability of *in vitro* blood-brain-barrier (BBB) model.

**Methods** An *in vitro* BBB model, established by co-culturing brain microvascular endothelial cells (BMVEC) and astroglial cells (AC) isolated from rat brain, was exposed to EMP at 100 kV/m and 400 kV/m, respectively. Permeability of the model was assayed by measuring the transendothelial electrical resistance (TEER) and the horseradish peroxidase (HRP) transmission at different time points. Levels of BBB tight junction-related proteins were measured at 0, 1, 2, 4, 8, 12, 16, 20, 24 h after EMP exposure by Western blotting.

**Results** The TEER level was lower in BBB model group than in control group at 12 h after EMP, exposure which returned to its normal level at 24 h. The 24 h recovery process was triphasic and biphasic respectively after EMP exposure at 100 kV/m and 400 kV/m. Following exposure to 400 kV/m EMP, the HRP permeability increased at 1-12 h and returned to its normal level at 24 h. Western blotting showed that the claudin-5 and ZO-1 protein levels were changed after EMP exposure.

**Conclusion** EMP exposure at 100 kV/m and 400 kV/m can increase the permeability of *in vitro* BBB model and BBB tight junction-related proteins such as ZO-1 and claudin-5 may change EMP-induced BBB permeability.

Key words: Electromagnetic pulse; Blood-brain-barrier; Permeability; TEER; HRP; ZO-1; Claudin-5

Biomed Environ Sci, 2013; 26(2):128-137	doi: 10.3967/0895-3988.2	013.02.007	ISSN:0895-3988
www.besjournal.com(full text)	CN: 11-2816/Q	Copyright ©20	13 by China CDC

#### INTRODUCTION

The increasing use of electromagnetic field generating equipments, such as mobile phone, computer, television, radio, and other wireless devices, has led to more and more concerns about their potential impact on health, particularly the effect of electromagnetic field exposure on human brain. A recent study implicated that radio frequency field exposure from mobile telephones increases the incidence of brain tumor<sup>[1]</sup>, which needs to be confirmed. Besides, other studies showed that EMP exposure can change the phenotype and electroencephalogram in animals<sup>[2-4]</sup>.

Received: December 22, 2011;

medicine.

Accepted: June 1, 2012

<sup>\*</sup>This work was supported by the National Basic Research Program of China (2011CB503704, 2011CB503705) and National Natural Science Foundation of China (No. 30970670, 60871068).

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EMP is a short and high-voltage pulse with an extremely rapid rise time and characterized by spectral bandwidth ranging from 0 Hz to 1.5 GHz. EMP is widely used in military campaigns and osteoporosis therapy. Such a relatively new technology has grown, but our knowledge is poor about its biological and potential harmful health effects on humans, especially on those who work in an electromagnetic radiation environment. Early studies demonstrated that EMP can detrimentally affect the endocrine system, circulatory system, and urinary system. However, its mechanism underlying damage to other organs such as brain still remains largely unknown<sup>[5]</sup>.

Blood-brain barrier (BBB) was discovered by E.E. Goldman in 1919 and its anatomical structure was confirmed by electron microscopy in the 1960s. BBB is essential for maintaining the internal environment homeostasis of brain in humans. The key functioning site is the tight junction (TJ) between endothelial cells (EC) lining the brain microvessels<sup>[6]</sup>, which excludes almost all exogenous materials from brain necessary for its normal function. Recent studies revealed that BBB has a dynamic structure consisting of EC, astrocytes, pericytes, perivascular microglia, and basement membrane.

*In vivo* studies showed that many factors can adversely affect BBB and its damage is closely related to TJ disruption<sup>[7-9]</sup>. However, no *in vitro* methods are available for quantitative analysis of EMF-induced BBB permeability changes. Such a model would facilitate detail studies on the molecular and functional changes of BBB in a controlled and manipulative environment.

In this study, an *in vitro* BBB model was established, and BBB permeability changes following exposure to EMP were detected using transendothelial electrical resistance (TEER) and horseradish peroxidase (HRP) transmission with its effect on the key TJ-related proteins, ZO-1, occludin, and claudin-5 assessed. The BBB model exhibited significant changes following exposure to EMP, thus providing an important manipulative environment for the assessment of EMP effect on BBB integrity.

#### MATERIALS AND METHODS

#### Animals

One-month old Sprague-Dawley female rats weighing  $90\pm10$  g and postnatal pups (1-2 d) were obtained from Animal Center of Fourth Military

Medical University (Xi'an, China). All procedures involving animals were performed in compliance with the China Animal Management Rule (Documentation No. 55; 2001, Chinese Ministry of Health). The rats were housed in separate cages with a specific pathogen-free laboratory environment at a temperature of 20-24 °C and a relative humidity of 40%-60% in a 12 h light/dark cycle with free access to sterile food and water.

# Cell Culture

Brain microvascular endothelial cells (BMVEC) were isolated from adult rats as previously described<sup>[10-12]</sup>. Meninges were carefully removed and gently rolled over filter paper to remove the white matter. Gray matter samples were cut into approximately 1 mm<sup>3</sup> sections which were submerged in D-Hanks solution. Collagenase type II (0.1 mg/mL; Sigma-Aldrich, MO, USA) and DNase (30 IU/mL; Roche Applied Sciences, Basel, Switzerland) were added to facilitate digestion, the mixture was incubated for 1.5 h at 37 °C. Then, 20% bovine serum albumin (BSA; Genview Corp., TX, USA) was added, the cell pellet was separated by centrifugation at 1000×g for 20 min at 4 °C. Microvessels were isolated from the pellet and further digested by incubation with collagenase-dispase (0.1 mg/mL; Roche Applied Sciences) and DNase (30 IU/mL) for 1 h at 37 °C. Clusters of endothelial cells in microvessels were separated by density gradient centrifugation in a 50% continuous Percoll solution (Sigma-Aldrich). BMVEC were collected, washed twice in phosphate buffered saline (PBS), and plated into 25 mm<sup>3</sup> culture flasks (Nunc Ltd., Roskilde, Denmark) precoated with 1% gelatin (Sigma-Aldrich), cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Corp., MD, USA) containing 20% fetal bovine serum (FBS; Gibco Corp.), basic fibroblast growth factor (bFGF; 1 ng/mL; Invitrogen Corp., CA, USA), heparin sodium (100 IU/mL; Hebei Changshan Biochemical Pharmaceutical Co. Ltd., Hebei, China), L-glutamine (2 mol/mL; Sigma-Aldrich), penicillin (100 IU/mL; Sigma-Aldrich), and streptomycin (100 IU/mL; Sigma-Aldrich) at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. The culture medium was changed every two days. When the cells reached 80% confluence (approximately eight to nine days later), purified endothelial cells were passaged in a 0.25% trypsin-0.02% EDTA (Sigma-Aldrich) solution. The presence of endothelial cells was confirmed with immunostaining of factor VIII (anti-human factor VII, 1:100 dilution; Zhongshan

Goldenbridge Biotechnology Co., Ltd., Beijing, China) and CD31 (anti-CD31, 1:200; Boster Biological Technology, Ltd., Wuhan, China).

Cerebral astrocytes were isolated from the neonatal pups as previously described<sup>[13]</sup>. Meninges were removed and gently rolled over filter paper, followed by additional scissor cutting so as to remove any remaining white matter. The gray matter was mechanically separated in D-Hanks solution. Brain tissue sections were digested by incubation in trypsin (0.25%) for 15 min at 37 ?C and passed through a 75 µm filter. The filtrate was collected and washed twice in PBS. The cells were seeded into cell culture flasks at a density of  $1 \times 10^{5}$ /cm<sup>3</sup> in a DMEM/F12 medium (Gibco Corp.,) containing 15% FBS, L-glutamine (2 mol/mL), penicillin (100 IU/mL), and streptomycin (100 IU/mL), and cultured at 37 ?C in a humidified atmosphere of 5%  $CO_2/95\%$  air. Pure astrocytes were obtained by sequential passaging. The cell culture medium was transferred into a new flask and cultured for an hour, during which, the fibroblasts adhered to the flask and the astrocytes remained free in the medium. Sequential transfer of the medium to a new flask and incubation removed all fibroblasts from the free cells, yielding pure astrocytes. The culture medium was changed every two days until the cells reached 80% confluency (approximately seven to eight days later). Flasks with confluent culture were incubated at 37 °C for 22 h with gentle agitation in а constant-temperature shaker (ZD-85 Guohua Co., Ltd., Changzhou, China) at 220 r/min. The medium was refreshed so as to remove the non-adherent oligodendrocytes and microglia. The purity of astrocytes was identified with immunostaining of glial fibrillary acidic protein (GFAP; Boster Biological Technology, Ltd., Wuhan, China).

# Establishment of In Vitro BBB Model

The *in vitro* BBB model was established by co-culturing BMVEC and astrocytes as described by Hurst et al.<sup>[14]</sup>. The astrocytes were seeded at a density of  $5 \times 10^5$ /cm<sup>3</sup> onto the undersurface of precoated (1% matrigel) transwell membrane inserts (12 mm diameter with 1 µm pore size and 1.12 cm<sup>2</sup> surface areas; Millipore Co., MA, USA). After the inserts were incubated in an upside down position for 12 h, they were returned to the upright position, and incubated until 80% confluency was reached. The BMVEC were seeded inside the insert at a density of  $1 \times 10^6$ /cm<sup>3</sup>. The TEER ( $\Omega$ cm<sup>2</sup>) was measured daily using the Millicell-electrical

resistance system (ERS) (Millipore Co.) until its absolute value reached 300  $\Omega$ cm<sup>2</sup>. The TJ was observed under S-3400N scanning electron microscope (SEM) (Hitachi Ltd., Tokyo, Japan) and JEM-2000EX transmission electron microscope (TEM) (Jeol Ltd., Tokyo, Japan) respectively with silver staining as reported by Raub et al.<sup>[15]</sup>. The model was considered successful if the TJ was observed under SEM and TEM with silver staining and its function was indirectly measured by TEER. When the TEER reached over 300 cm<sup>2</sup>, the established model was considered valid and used in further experiments<sup>[16]</sup>. The procedure is shown in Figure 1.

Change the insert to normal position



**Figure 1.** Procedures for the establishment of BBB model. AC were plated underneath the transwell insert, the insert was transferred to the upright position 12 h later. Once AC reached 80% confluency, BMVEC were seeded inside the insert. TEER was measured daily until >300  $\Omega$ cm<sup>2</sup> was reached. Tight junctions were confirmed SEM and TEM with silver staining.

# EMP Exposure

EMP (peak-intensity 400 kV/m, rise-time 10 ns, pulse-width 350 ns, 0.5 pps, 400 pulses total) was generated by a spark gap pulse generator and transmitted into а Gigahertz transverse electromagnetic (GTEM)-cell. Both EMP generator and GTEM-cell were devised by the Department of Mechanical Engineering at Southeast University (Nanjing City, Jiangsu province, China). The EMP waveform is shown in Figure 2. In order to observe the dose-effect of EMP exposure, the cells were divided into different groups and exposed to EMP at different intensities. The cells were exposed or sham-exposed to EMP for 100, 200, or 400 pulses at 100 kV/m and 400 kV/m, respectively. After exposure, no significant change occurred in the temperature of medium. The cells in sham group were placed in the similar exposure chamber but not exposed to EMP.



**Figure 2.** Waveform of EMP in BBB model. The EMP (peak-intensity 400 kV/m, rise-time 10 ns, pulse-width 350 ns, 0.5 pps, 2000 pulses, and the average SAR of 0.04 mW/mL) was generated by a spark gap pulse generator and transmitted into a Gigahertz transverse electromagnetic cell.

## Measurement of TEER

The BBB permeability was assayed by measuring the TEER across the cell monolayer. TEER represents the impedance required to pass through the barrier structure, and is widely recognized as one of the most accurate and sensitive indicators of BBB integrity<sup>[17-18]</sup>. A decrease in TEER reflects an increase in permeability and a loss of barrier function. In this study, TEER of the BBB model was recorded in PBS at 0, 1, 2, 4, 8, 12, 16, 20, and 24 h after EMP exposure using the Millicell-ERS. The resistance value was multiplied by the surface area of the insert  $(1.12 \text{ cm}^2)$ and expressed as  $\Omega \text{cm}^2$ . The TEER of each sample was corrected for background resistance without cells and reported as  $\Omega cm^2$ . The results were normalized to TEER measured before EMP exposure and presented as absolute values.

## Measurement of HRP Transmissivity

In this study, horseradish peroxidase (MW 40 kD, Sigma-Aldrich) transmissivity was measured as previously described with slight modifications<sup>[19-20]</sup>, in order to determine the *in vitro* BBB permeability to micromolecular materials. After the EMP exposure for 0, 1, 2, 4, 8, 12, 16, 20, 24 h, the culture medium was replaced with DMEM without phenol red (Gibco Corp.) or serum. In order to maintain the liquid level, 460  $\mu$ L medium containing 500 ng HRP was added into the insert, and 1140  $\mu$ L medium was added into the well. A total of 50  $\mu$ L medium was

collected from each well at different time points, and 50  $\mu\text{L}$  fresh medium was added after each collection in order to maintain the liquid level on both sides. The collected samples were stored at 4 °C until processing while 100 µL peroxidase substrate containing tetramethyl benzidine and hydrogen peroxide was added to each sample and incubated for 3 min. The reaction was terminated by adding 50  $\mu$ L sulphuric acid (1 mol/L). The optical density was measured at 450 nm and the HRP transmissivity was assayed from the standard curve according to the following equation:  $T_{HRP}\% = [(C_{HRPo} \times V_o / C_{HRPi} \times V_i)]$  $\times$ 100%], where C<sub>HRPo</sub> is the HRP concentration in the well,  $C_{HRPi}$  is the HRP concentration in the insert,  $V_o$  is the medium volume in the well, and  $V_i$  is the medium volume in the insert.

#### Measurement of Tight Junction-related Proteins

At the post-exposure time points mentioned above (before EMP exposure and 0, 1, 2, 4, 8, 12, 16, 20, 24 h after EMP exposure), brain microvascular cell monolayers were washed in ice-cold PBS and collected by scraping. Samples were immediately frozen and stored at -20 °C until processing while total protein samples were collected by disrupting the mechanical cells for Western blotting analysis. Proteins were separated by SDS-PAGE and transferred to polyvinylfluoride membranes (Millipore Co.,). Non-specific binding sites were blocked by incubating with 5% dried skimmed milk (diluted with Tris-buffer solution supplemented with Tween (TBST)) at room temperature for 3 h. Blots were incubated with anti-claudin-5 or anti-occludin mouse monoclonal antibodies, or anti-ZO-1 rabbit polyclonal antibody diluted at 1:800 (Zymed Laboratories Inc., CA, USA) at 4 °C for overnight. Peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins (Zhongshan Goldenbridge Biotechnology Co., Ltd.) were applied as secondary antibodies. After incubation, blots were washed 3 times with TBST, 15 min each time. Immunoreactive bands were detected by incubating the blots with chemiluminescent HRP substrate (Millopore Corp.) and imaging with the Chemidoc XRS System (Bio-Rad Laboratories, Segrate, Italy).

# **Statistical Analysis**

Data were analyzed, using SPSS statistical software package (version 16.0; SPSS, Inc., IL, USA) by Student's *t*-test or one-way analysis of variance (ANOVA). Results were expressed as mean $\pm$ SD. *P*<0.05 was considered significant. The number of

parallel inserts was 6 and all experiments were repeated at least 3 times.

# RESULTS

# Establishment of In Vitro BBB Model

The astrocytes were isolated from rats. Immunocytochemical labeling showed that the astrocytes expressed the GFAP astrocytic marker (Figure 3A-B). Following passage, >98% of astrocytes were GFAP positive. The BMVEC were confirmed by immunocytochemistry to detect factor VIII (Figure 3C-D) and CD31 (Figure 3E). After the first transfer of BMVEC culture, 95% purity was achieved. In the co-culture model, BMVEC formed a confluent monolayer at approximately 7-9 days after plating. The widely formed TJ and the foot process of astroglial cells also reached the side of BMVEC monolayer and crossed into the micropore filter, as under SEM and TEM evidenced with silver staining (Figure 3F-I).



**Figure 3.** Primary culture and identification of brain microvascular endothelial cells and astroglial cells. (A) Phase contrast image of astroglial cells after first transfer of culture, (B) GFAP immunostaining of AC, (C) Photomicrograph showing primary culture of BMVEC, (D) Factor VIII-labeled BMVEC, (E) CD31-labeled BMVEC showing the morphology of tight junctions, (F) Silver nitrate staining showing widely formed TJs (black filaments) between nearby BMVEC, (G, H) SEM and TEM of TJs, (I) The foot processes of AC crossing the micropores in the filter and reaching the BMVEC on the other side.

The permeability to ions and low molecular weight molecules was assessed daily by measuring the TEER. The TEER increased after cultured for 6 days (Figure 4). Then, the TEER was significantly greater in co-cultures than in monocultures that were grown for the same time period. The TEER reached a plateau after co-cultured for 8 and 9 days, during which the TEER was  $65.36\pm7.5 \ \Omega cm^2$  on day 6 (*n*=4) and  $353.02\pm7.5 \ \Omega cm^2$  on day 9 (*n*=4). The TEER was significantly higher than the electrical resistance in astrocytes (131.86±8.08  $\Omega cm^2$ , 41.80±1.53  $\Omega cm^2$ , *P*<0.05). Our results are consistent with those of a related study<sup>[21]</sup>, suggesting that co-culture plays an important role in the formation of tight BBB.

# **TEER Change**

Following EMP exposure, the TEER of *in vitro* BBB model became unstable, tended to decrease 12 h after EMP exposure, and returned to normal 24 h after EMP exposure (Figure 5). As shown in Figure 5A, after EMP exposure at 100 kV/m, 3 distinct recovery phases occurred at 0 h,  $4^{8}$  h, and 12 h. After EMP exposure at 400 kV/m, only one

recovery phase occurred at 24 h (Figure 5B). The other two recovery phases were similar to that after EMP exposure at 100 kV/m. The TEER of the control group remained unchanged at all time points



**Figure 4.** TEER in different cell layers. EMP exposure- produced similar TEER in co-culture cells and the two monocultured cell layers on day 6, exponential increase of TEER in co-cultured cells, and exceeding  $300 \ \Omega \text{cm}^2$  within 3 days (\*P<0.05).



**Figure 5.** Alterations in TEER in response to different EMP exposure conditions. (A) Response to EMP exposure at 100 kV/m showing 3 distinct and immediate recovery processes within 24 h, (B) Response to EMP exposure at 400 kV/m showing only two recovery processes at 24 h after EMP exposure, which was similar to that after EMP exposure at 100 kV/m except for instantaneous recovery process ( $^*P$ <0.05).

## HRP Permeability Change

The permeability to low molecular weight molecules was assessed by measuring the permeability coefficient of HRP (342.3 D). As shown in Figure 6, the HRP permeability increased 12 h after exposed to EMP at 400 kV/m and then returned to normal levels and no significant difference was found in HRP permeability between BBB model group and control group after exposed to EMP at 100 kV/m.

## EMF-induced Effects on TJ-related Proteins

Brain endothelial cells were investigated at different time points following exposure to different electromagnetic pulses. The levels of TJ proteins, including occludin, claudin-5, and ZO-1 measured by Western blotting analysis, are shown in Figure 7. The claudin-5 levels decreased from 0-12 h after EMP exposure and gradually returned to its normal level at 24 h after EMP exposure, which is similar to that of TEER. The ZO-1 level was significantly different



**Figure 6.** HRP transmissivity in response to EMP exposure. The HRP transmissivity increased gradually 12 h after EMP exposure at 400 kV/m and returned to its normal level at 24 h. No change in HRP transmissivity was observed after EMP exposure at 100 kV/m ( $^{*}P$ <0.05).

following exposure to EMP, which is similar to that of TEER except for the recovery at 0 h after EMP exposure at 100 kV/m.

#### DISCUSSION

Although the potential biological effects of EMP exposure on isolated cells and whole animals were studied, no consensus on whether the effect is thermal or non-thermal has been reached. It was reported that the main biological effect of EMP exposure is thermal<sup>[22]</sup>. It has been demonstrated that the effect of EMP is non-thermal<sup>[23-26]</sup>. In our study, the EMP exposure to BBB is non-thermal because no temperature change in culture medium was observed.

Whether the effect of EMP exposure is positive or negative is still controversial. It was reported that some subjects are completely unaffected after EMP exposure<sup>[27-30]</sup>. The positive effect of EMP exposure focuses on the factors about cartilage, but most studies showed that effect of EMP exposure is negative<sup>[31-33]</sup>.

It has been confirmed that EMF can disturb the barrier function of BBB<sup>[26,34-38]</sup>. However, Grafström et al reported that no histopathological change occur in rat brain following long-term EMP exposure or GSM-900 mobile phone radiation<sup>[39]</sup>.

In our previous study, exposure to 200 and 400 pulses (1 Hz) of EMP at 200 kV/m could increase the cerebral microvascular permeability in rats, which can be resolved over time or inhibited by some factors<sup>[9,40-41]</sup>. Since no quantitative analysis of the BBB permeability was conducted, An *in vitro* BBB

model was established in this study to measure the dose-and time-effect of EMP exposure on BBB permeability. Since BBB function is known to depend upon the tight junctions between neighboring BMVEC

and can be induced by astrocytes, these two cell types were co-cultured to establish an *in vitro* BBB model that is more closely simulated the *in vivo* situation as described by Panula et al.<sup>[42]</sup>.





**Figure 7.** TJ-related protein expression change in brain endothelial cells after EMP exposure. The upper panels indicate representative immunoblots of ZO-1, occludin and claudin-5. Their semi-quantified data are shown in the lower panel. Each value is expressed in percentage. Data represent mean $\pm$ SD (*n*=6). \**P*<0.05 is considered significant. Occludin did not change in any situation. Claudin-5 decreased at first and then increased 24 h after EMP exposure. ZO-1 decreased at first in a fluctuating manner, and then steadily increased 24 h after EMP exposure.

Using the *in vitro* BBB model, the two indices of BBB permeability and TEER were measured after EMP exposure as described in other studies<sup>[16-17,22]</sup>. The TEER decreased at first, then began to recover at 12 h after EMP exposure, and returned to its normal level at 24 h after EMP exposure. Variations in radiation intensity and pulse time caused differences in the time-course of TEER change. Three distinct recovery processes were observed 24 h after exposure to EMP at 100 kV/m. However, only two distinct recovery processes were observed 24 h after EMP exposure at 400 kV/m. The self-repair mechanism was inhibited with the increasing radiation intensity. The TEER returned to its normal level at 24 h after EMP exposure and remained stable thereafter (data not shown).

The typical method to confirm the BBB barrier function is to measure <sup>14</sup>C-sucrose, <sup>125</sup>I-BSA HRP, *etc.* In this study, the hydrosoluble HRP molecule (relative molecular mass: 40 kD) as a marker of BBB permeability was not detected. EMP exposure at 400 kV/m caused increased the HRP transmission rate significantly. However, no significant change was observed in HRP transmission rates at any of the

other time points. We inferred that although EMP exposure caused the BBB barrier function change, the effect of EMP exposure on BBB permeability was limited. Only the strongest radiation intensity could transfer HRP to BBB after EMP exposure, and weak exposure conditions could not make the aperture large enough. The results can confirm the findings in our previous study<sup>[9,40-41]</sup>.

In addition, the mechanism underlying BBB permeability change was studied in this study. It is known that BBB function is dependent on tight junctions which are constructed by an array of specialized proteins, including ZO-1, claudin-5, and occludin. The ZO-1, claudin-5, and occludin levels were measured at different time points after EMP exposures. Western blotting analysis showed that the occludin-5 levels did not change at any time point or after any EMP exposure, indicating that claudin-5 does not play any role in EMF-induced BBB damage and its repair. Claudin-5 levels, however, were reduced after EMF exposure. The lowest claudin-5 level was observed at 12 h after EMP exposure and returned to its normal level at 24 h, which agreed with the general trend of TEER, especially for the recovery 12 h after EMP exposure. Interestingly, the changes in ZO-1 levels followed the early and later recovery phases. Therefore, both ZO-1 and claudin-5 appear to play a role in the recovery process at 12 h, but only ZO-1 plays a role in the early recovery process. We therefore considered that EMP exposure could affect the BBB barrier structure, allowing small ions to pass through and triggering different repair mechanisms at different time points after EMP exposure. However, some unknown immediate repair mechanisms were inhibited with the increase in radiation intensity, for which further study is needed.

We highly appreciate Professor Kevin M. PRISE from Centre for Cancer Research & Cell Biology of Queen's University Belfast for his valuable advice.

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