

Effects of Universal Mobile Telecommunications System (UMTS) Electromagnetic Fields on the Blood-Brain Barrier in Vitro

H. Franke^{*1}, J. Streckert², A. Bitz², J. Goeke³, V. Hansen², E.B. Ringelstein¹ and F. Stögbauer¹

¹ Klinik und Poliklinik für Neurologie, Universitätsklinikum Münster, Münster, Germany

² Lehrstuhl für Theoretische Elektrotechnik, Bergische Universität Wuppertal, Wuppertal, Germany

³ Institut für technische Gebäudeausrüstung, Fachhochschule Köln, Cologne, Germany

*corresponding author:

Helmut Franke, Klinik und Poliklinik für Neurologie, Albert-Schweitzer-Str. 33, 48129 Münster,
Germany, Phone: +49-251-83-48329, Fax:+49-251-83-48181, Mail: hfranke@uni-muenster.de)

keywords:

blood-brain barrier, in vitro, mobile telephone communication, permeability, electrical resistance

running title:

RF-EMF effects on the blood-brain barrier in vitro

total word count: 6,386

Abstract

The extensive use of mobile phone communication has raised public concerns about adverse health effects of high frequency electromagnetic fields (EMF) in recent years. A central issue in this discussion is the question whether EMF enhance the permeability of the blood-brain barrier (BBB). Here we report an investigation on the influence of a generic UMTS (Universal Mobile Telecommunications System) field on barrier tightness, transport processes and the morphology of porcine brain microvascular endothelial cell cultures (PBEC) serving as in vitro model of the BBB. An exposure device with integrated 'on-line' monitoring system was developed for simultaneous exposure and measuring of transendothelial electrical resistance (TEER) to determine BBB tightness. PBEC were permanently exposed for up to 84 h at an average electric field strength of 3.4-34 V/m (max. 1.8 W/kg) assuring athermal conditions. We did not find any evidence of EMF-induced disturbance of BBB function. After and during exposure, BBB tightness quantified by ¹⁴C-sucrose and serum albumin permeation as well as by TEER remained unchanged compared to sham exposed cultures. Permeation of transporter substrates at the BBB as well as the localization and integrity of the tight junction proteins occludin and ZO-1 were not effected either.

Introduction

The blood-brain barrier (BBB) is a particularly interesting system regarding effects of high-frequency electromagnetic fields (EMF) on biological systems, because of its vicinity to the radiation source and its vital function in the human brain. This function is to maintain the homeostasis of the cerebral microenvironment which is essential to neuronal function and activity.

The mammalian BBB is built up by brain capillary endothelial cells lining the cerebral capillaries. The cleft between adjacent endothelial cells is sealed by tight junctions (TJ), a specific type of intercellular contacts found in epithelia as well as endothelia (Tsukita et al., 2001). TJ form continuous circumferential contacts between lateral plasma membranes and thus prevent free diffusion of solutes via the paracellular pathway. Although it is recognized that proteins are not the only structural basis

responsible for the development of TJ (Wegener and Galla, 1996), the interplay of several proteins at the site of TJ is crucial for barrier formation.

The participation of the transmembrane proteins occludin (Furuse et al., 1993), claudin-1 and claudin-5 (Furuse et al., 1998) as well as the cytosolic peripheral membrane proteins ZO-1 (Stevenson et al., 1986), ZO-2 (Jesaitis and Goodenough, 1994) and ZO-3/p130 ((Balda and Anderson, 1993; Haskins et al., 1998)) in TJ formation is generally accepted. Occludin was the first integral TJ protein to be identified (Ando-Akatsuka et al., 1996; Furuse et al., 1993). It consists of four transmembrane domains, a short N-terminal and a long C-terminal cytoplasmic domain. The latter is directly bound to the peripheral TJ proteins ZO-1, ZO-2 and ZO-3 (Furuse et al., 1994; Haskins et al., 1998; Itoh et al., 1999a; Itoh et al., 1999b; Stevenson et al., 1986), indicating that occludin annexes these cytoplasmic proteins to the membrane. The presence of occludin at TJ is accompanied by enhanced barrier function and decreased paracellular permeability (Hirase et al., 1997). Intracellular proteins ZO-1, ZO-2 and ZO-3 are concentrated at the cytoplasmic side of TJ, very close to the plasma membrane, and are assumed to anchor integral plasma membrane proteins like occludin to the actin cytoskeleton. They might form a scaffold for transcription factors and signaling proteins involved in regulation of cell proliferation and differentiation (D'Atri and Citi, 2002). Balda and Matter (Balda and Anderson, 1993) found indirect evidence for a regulatory function of ZO-1 in barrier formation, emphasizing a pivotal role of this protein for the differentiation of barrier-forming cells.

The tightness of TJ and thus the integrity of a cellular barrier can be directly measured as transepithelial or transendothelial electrical resistance (TEER) which reflects the permeability of TJ for small ions and solutes (Schneeberger and Lynch, 1992). A convenient method of assessing the TEER is by means of impedance analysis as described in detail by Wegener (Wegener et al., 2000; Wegener et al., 1996). In vivo, cerebral microvascular endothelial cells develop high TEER of 1500–2000 $\Omega\cdot\text{cm}^2$ (Butt et al., 1990). Cell culture models of the BBB show significantly lower TEER values. For a comparison, human umbilical vein endothelial cells (HUVEC) as an example of macrovascular endothelial cells develop in vitro TEER of only about 5–10 $\Omega\cdot\text{cm}^2$ (Keese et al., 2002). We have previously developed a well-characterized in vitro model of the BBB, based on primary cultured

porcine brain capillary endothelial cells (Engelbertz et al., 2000; Franke et al., 1999; Franke et al., 2000; Hoheisel et al., 1998). Withdrawal of serum from confluent cultures guarantees highly standardized experimental conditions and improves barrier properties, resulting in TEER values of up to $1000 \Omega\text{-cm}^2$. Additionally, this BBB model displays a very low paracellular permeability e.g. for sucrose a commonly used marker compound $3.4 \cdot 10^{-7} \text{ cm/s}$ (Lohmann et al., 2002). These data indicate a very tight and intact cellular barrier with well developed TJ that closely mimics the in vivo situation ($1500\text{--}2000 \Omega\text{-cm}^2$ (Butt et al., 1990) and $1.2 \cdot 10^{-7} \text{ cm/s}$ (Levin, 1980) respectively).

As the tight junctions completely seal the paracellular cleft between adjacent brain capillary endothelial cells, transfer of solutes across the brain endothelium mainly occurs transcellular. Numerous transport mechanisms are present at the cell membranes that are essential for the adequate supply of the CNS with nutrients. D-glucose as the main source of energy for the brain is a water-soluble substance that enters the brain via facilitated diffusion. Glucose transport into the brain occurs via the GLUT-1 protein (Crone, 1965) and is highly stereospecific for D-glucose. The driving force for glucose transport is the concentration gradient of the compound from blood (high conc.) to brain (low conc.). In contrast to this facilitated diffusion, active transport systems transfer substrates from regions of lower to regions of higher concentration dependent on ATP. For example, the A-system (alanine-preferring transporter) localized in the abluminal membrane of BEC ensures the transport of small neutral amino acids out of the brain into the endothelial cell (Goldstein and Betz, 1986), protecting the CNS from high concentrations of glycine which is a potent inhibitory neurotransmitter.

The L-system (leucine-preferring transporter), responsible for the transport of larger amino acids, is localized both on the luminal and the abluminal side of the endothelium contributes to the maintenance of homeostasis in the brain tissue.

It is still an open question whether the tightness of the blood-brain barrier (BBB) is affected by weak electromagnetic fields, e.g. by radio frequency (RF) -fields derived from digital mobile communication systems. Results of animal experiments dealing with this issue (Finnie et al., 2002; Finnie et al., 2001; Persson et al., 1997; Salford et al., 1994; Tsurita et al., 2000), are discussed

controversially because experimental approaches are yet not sufficiently well established and cannot exclude influences like are stress of test animals and inter-individual variations on the results of the influence of electromagnetic fields. In this context, Fritze et al. (Fritze et al., 1997) observed increased BBB permeability even in sham-exposed rats in comparison to cage control animals, an effect that might be stress-attributed since the rats are restrained plastic tubes during (sham) exposure.

Materials and Methods

Isolation and culture of PBEC

Brain capillary endothelial cells were used as primary cultures. This means they were either freshly prepared from porcine brains or thawed from nitrogen frozen stocks of the same. PBEC were isolated from freshly slaughtered pigs' brains as described previously (Franke et al., 2000). In brief, meninges and choroid plexus were removed from the brains and cerebra were homogenized mechanically. The homogenate underwent digestion in 1% (w/v) dispase followed by density gradient centrifugation to obtain a pellet of the released capillary fragments. Endothelial cells were released from the capillaries by a second digestion with collagenase, breaking up the collagen matrix surrounding the vessel. PBEC were collected from the interface of a percoll density gradient, plated in collagen G coated culture flasks and cultivated in M199 medium supplemented with 10% newborn calf serum (PAA, Coelbe, Germany), 0.7 mM L-glutamine and antibiotics: 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml gentamicin. Twenty-four hours after initial plating, cells were washed with phosphate buffered saline (PBS) and supplied with fresh culture medium. For further purification, PBEC were subcultivated at day 3 by fractionated trypsination. This enzymatic treatment selectively released endothelial cells, reducing the number of contaminating cells such as pericytes and smooth muscle cells. Purified endothelial cells were then sown on rat-tail collagen coated Transwell® cell culture inserts (1.13 cm² surface area, 0.4 µm pore-size, Costar, Bodenheim, Germany). Surplus cells were frozen in liquid nitrogen and used as a stock for further experiments.

PBEC on filter inserts were grown for three days in the same medium as used before passage. Then the medium was changed to serum-free DMEM/Ham's F12 supplemented with L-glutamine and

antibiotics as described above. In contrast to previous reports (Franke et al., 1999; Hoheisel et al., 1998) no hydrocortisone was added.

All cell culture media and supplements were obtained from Biochrom (Berlin, Germany) unless otherwise noted. Other chemicals were obtained from commercial sources in the highest quality available.

Exposure of BBB-cell cultures to EMF

PBEC cultures were exposed to a generic 1.966 GHz UMTS signal (Ndoumbè Mbonjo Mbonjo et al., 2004) at field strengths ranging from 3.4-34 V/m in an implemented exposure system, assuring non-thermal conditions. The UMTS signal originated from a signal generator GUS 6960S (University of Wuppertal). TEER values were recorded by means of impedance spectroscopy (IS). This method allowed a permanent, non-invasive monitoring of barrier tightness during exposure to RF electromagnetic fields. The exposure unit consisted of two radial waveguides, each containing up to 28 samples, a field- and a thermistor probe. An overview of the exposure system is given in Fig. 1. Two waveguides were placed into an incubator and could either in turn be used as exposure or sham exposure devices. The selection between exposed and sham-exposed waveguide was controlled by a software for each individual experiment. A protocol of the actual exposure setting was written into an encrypted file on a personal computer. Thus it was guaranteed that the experiments were double-blinded. Each sample holder was equipped with a two electrode system in order to allow for individual impedance monitoring. The excitation of the RF-field was achieved via a shape-optimized antenna in the center of the waveguide. A 5 mm flat absorber (Emerson & Cuming, ECCOSORB SF-U2.0) along the waveguide perimeter reduces reflections substantially. The samples in each waveguide were arranged symmetrically near the rim. The leads of the electrodes were conducted upwards through metallic caps closing the cartridges. Thereby, simultaneous RF exposure and LF impedance measurement was possible at any time of the experiment. For exposure experiments, the filter inserts carrying the cell cultures were placed in cylindrical polycarbonate tubes specially designed for this study (Fig. 2) to fit exactly into the waveguides cavities. For TEER recording purposes a counter electrode was placed into every tube below the filter insert.

5 mm above every filter membrane, a gold ring surrounded the filter insert. The upper electrodes were mounted into caps, which worked as plug connectors for the wires leading to the impedance spectrometer. Fig. 2 shows all individual parts, the complete assembly was mounted as seen in Fig. 3. The tubes were positioned inside the waveguides and locked with a cap nut. The upper electrodes and rings were designed to be in plane with the upper disk of the waveguide, as was the counter electrode with the lower disk. This allowed propagation of the RF-field in the electrodes and ring, yielding optimized field distribution and field homogeneity at the cell monolayer. To avoid interference of RF signal and LF field of the resistance measurements, a capacitor was mounted in the cap of the apical electrode to serve as low pass filter.

Transendothelial electrical resistance

The barrier tightness was permanently monitored during RF exposure by TEER measurement using impedance spectroscopy (described in detail by Wegener (Wegener et al., 2000)). Briefly, the filters carrying the cell cultures were installed as described above between two discoid gold electrodes. Impedance analysis was carried out in the frequency range from 1 Hz to 500 kHz applying a sinusoidal alternating voltage of ~30 mV amplitude (arbitrary function generator AFG 310, Sony Tektronix, Cologne, Germany; Multimeter/Switch System Model 2760, Keithley, Germering, Germany) under normal cell culture conditions (37 °C and humidified atmosphere of 5% CO₂ /95% air). The TEER was calculated from recorded impedance spectra as follows.

Considering the expression for the absolute value of the impedance Z_C of the cell layer,

$$|Z_C| = \sqrt{\left[\frac{TEER}{1 + (\omega TEER TEEC)^2} \right]^2 + \left[\frac{\omega TEER^2 TEEC}{1 + (\omega TEER TEEC)^2} \right]^2}$$

we distinguished between two cases according to the dependence of frequency.

At low frequencies the contribution of the capacitance $TEEC$ of the cell layer was negligible and we obtained a simplified relation,

$$|Z_c| = TEER \quad \text{on condition that } \omega * TEER * TEEC \ll 1.$$

The condition was precisely met in a range of 60 Hz to 600 Hz. Thus, the TEER of the PBEC layer including the filter membrane could be calculated from the average of impedances determined in this range. An average impedance spectrum of cell-free filter inserts was subtracted from each spectrum allowed to calculate the TEER for the pure cell layer from the difference of the systems impedance including cells and the impedance without the cell monolayer. This method allowed the convenient determination of the TEER of cell layers without any mathematical simulation.

Permeability assay

For transport studies, PBEC were removed from the waveguide and transferred into 12-well plates supplied with fresh serum-free medium in the basolateral compartment. Liquid levels in both compartments were adjusted to avoid hydrostatic pressure. Approximately 0.15 μCi of tracer (^{14}C -sucrose, ^3H -glucose, ^3H -leucin, ^3H -alanin, or ^{125}I -bovine serum albumin) were added to the apical compartment of each filter and mixed gently. Four samples were collected from the basolateral compartment at consecutive time points. After each sampling the volume in the acceptor compartment was replenished with fresh medium. Radiation was quantified by liquid scintillation counting. Cultures were discarded after a permeation experiment and not replaced into the waveguide. Permeation coefficients were calculated as reported earlier (Franke et al., 2000), averaged over a set of three to six filters and expressed as velocity (permeation coefficient P in $[\text{cm}/\text{s}]$).

Response of PBEC to thermal stress

The tolerance of PBEC towards thermal stress was examined by cultivating cells from identical preparation batches at different temperatures. For this purpose, PBEC were plated as usual on collagen coated filter inserts and maintained at 37°C for three days. After switching to serum-free medium, the cultures were divided into two groups, one being replaced into the incubator set to 37°C , the other

into an incubator with elevated temperatures in the range of 37.3-39.0° C. BBB tightness of both groups was monitored using the sucrose permeability assay on three consecutive days after the switch to serum-free medium. A control experiment was carried out with both incubators set to 37° C. Temperature elevation started at the same time point during culture as RF-exposure did in corresponding experiments.

Threshold for athermal exposure conditions

To determine the temperature increase in the culture medium in response to the applied RF field, the exposure unit was installed inside the incubator, including impedance electrodes and filter inserts but without cells. A self-calibrating thermometer “*Soliton FTI-10*” (Soliton GmbH, Gilching, Germany) with glass hollow fiber sensor “*FOT-M*” was inserted into a filter chamber through a ventilation drill hole and placed close to the filter surface. Temperature was monitored over time and recorded by a personal computer. After closing the incubator door, the system was allowed to equilibrate for several hours. As soon as temperature values showed a constant baseline, the RF-field was activated and the temperature change was recorded until equilibrium.

Simultaneous RF-exposure and impedance spectroscopy

A non-linear behavior of any component in the signal path would result in an interference of the RF-exposure signal with the LF impedance spectroscopy signal. However, neither the direct measurements of the LF current through the RF-exposed BBB model nor measurements of frequency components due to intermodulation by application of the ‘two-tone measuring technique’ with help of the respective calibration signal implemented in the GUS generator exhibited a hint for a possible non-linearity of the electronic components, the electrode system, the electrolyte or the BBB cell layer, respectively. Thus, LF and RF path can be regarded separately without any mutual impact disturbing the impedance measurement.

Calculation of field distribution and field strength

The calculation of the field distribution inside the exposure device was performed by numerical software tools based on the Finite Difference Time Domain (FDTD) method. Because of the stationary arrangement, it is sufficient to model a single sector of the radial waveguide with vessel. The boundary conditions in planes $\varphi = 0^\circ$ and $\varphi = 12^\circ$ are set to be ideal magnetic. The TEM-wave is excited at the inner radial boundary. This configuration models the complete radial waveguide with 30 vessels.

A non-uniform mesh with step sizes ranging from 0.25 mm to 2 mm is applied for the calculation. In the region of the insert with BBB-model a uniform step size of 0.25 mm is used.

For the numerical dosimetry the BBB-model is neglected, because there is no information available about the intrinsic permittivities and conductivities of the materials forming the blood brain barrier cell layer. According to the commonly applied approach for the estimation of the SAR for cell systems, the field strength and SAR across the cell layer are given as averaged values in the voxel layer of the FDTD-model which encloses the BBB-model in the vessel. Then, the averaging mass for the SAR based on a mass of a voxel of 0.0156 mg is c. 30 mg. Since the SAR describes a heat source in the unknown heat transfer system of the exposure device, measurements of the temperature increase due to the rf-exposure have to be performed in order to assure that the produced heat inside the vessel is dissipated through the vessel and, consequently, the temperature increase in the vessel is limited. Due to the volume to be considered the spatial resolution of the numerical computation is magnitudes larger than the dimensions of a biological cell (diameter, membrane thickness). Therefore, results of calculations as well as the results of measurements are only valid for the macroscopic region surrounding the cell layer and give no information about the microscopic SAR and temperature distribution inside the cells.

During the course of experiments two configurations have been used. For permeation experiments with sucrose the inserts were placed into vessels without electrodes, for impedance spectroscopy the vessels with electrodes were utilized.

Blinding of the experiment

All experiments were carried out double-blind. Exposed and sham-exposed waveguide were randomized at the beginning of the exposure by an automated switch. A protocol of the actual exposure setting was written into an encrypted file on a personal computer.

Monitoring of exposure parameters

The electromagnetic field and the temperature of the medium were constantly monitored during RF-exposure. A field antenna and a thermistor probe were each installed inside a cell culture vessel and data were recorded and displayed via Labview software (National Instruments, Munich, Germany) on a personal computer.

Immunocytochemistry

For indirect immunofluorescence studies, confluent PBEC grown on gelatin-coated glass cover slips were washed twice with PBS and fixed with methanol/acetic acid (95:5) for 10 min at -20 °C. After four subsequent washes with PBS, cells were soaked in blocking solution (3% (w/v) bovine serum albumin in PBS) for 30 min at 37 °C and subsequently incubated for 60 min with mouse anti-occludin antibody (1.25 µg/ml, diluted 1:400, Zymed (Berlin, Germany)) or rat anti-ZO-1 antibody (2.0 µg/ml diluted 1:600, Chemicon (Hofheim, Germany)) in PBS containing 0.5% (w/v) BSA.

Samples were washed four times with PBS, soaked again for 30 min in blocking solution at 37 °C and incubated with a dilution (2 µg/ml) of fluorophore-labelled secondary antibody (MoBiTec, Göttingen, Germany) in PBS containing 0.5% (w/v) BSA for 30 min at 37 °C. After three washes with PBS, cover slips were mounted in ProLong Mountingmedium (Molecular Probes, Leiden, The Netherlands).

Positive control experiments

Artificial opening of the BBB in vitro was achieved by applying either a 1.4 M solution of mannitol in PBS or by thermal stress. Mannitol solution was mixed 1:1 with the apical medium before permeation experiments. For thermal stress experiments the culture temperature was elevated to 45 °C and the change in TEER was recorded simultaneously.

Electrophoresis and Immunoblotting

Cells grown on rat tail collagen coated Transwell-Clear filter inserts were washed twice with PBS and lysed for 10 min with 20 µl lysis buffer (25 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 4 mM EDTA, 25 mM NaF, 1% (w/v) SDS), along with protease inhibitor cocktail for mammalian tissues (Sigma, Munich, Germany; concentration according to the manufacturer's instructions) at 4 °C. The lysate was heated to 90°C for 10 min, passed several times through a 25-G needle and treated with ultrasound.

SDS-PAGE was performed according to Laemmli (Laemmli, 1970). Cell lysate was loaded to an 8.0% (occludin) or 6.0% (ZO-1) SDS polyacrylamide gel. Following electrophoresis the proteins were transferred to an Immobilon P membrane (Millipore).

TJ proteins were detected by the use of polyclonal rabbit anti-ZO-1 (dilution 1:2000) and anti-occludin (dilution 1:3000) antibodies (Zymed, Berlin, Germany). The membranes were washed three times with 10 mM Tris-HCl pH 7.4, 150 mM NaCl and 0.05% Tween 20 (TBST) and then incubated with goat-anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibody (1:3000) for 1 h followed by three washes using TBST. Immunoreactive bands were detected by enhanced chemiluminescence (ECL-Kit, Amersham Pharmacia Biotech, Braunschweig, Germany) according to the manufacturer's instructions.

Results

Dosimetry

The electric field distribution across the BBB-model at a frequency of 1.966 GHz and for the time-averaged input power of 1.5 W is given in Fig. 4 as radial cut through the vertical axis of a vessel with the electrode system inserted.. On average an electrical field strength of 34 V/m with a standard deviation of 40% and a voxel layer averaged SAR of 1.8 W/kg (SD 82%) is achieved. The vessel-averaged specific absorption rate is 1.64 W/kg. The ratio of maximum and minimum field strength in

the cell layer under consideration of the complete environment is $\frac{|E|_{\max}}{|E|_{\min}} \approx 6$.

The insertion of the electrodes, in particular the electrode rod of the lower plate electrode, decrease the mean electric field across the cell layer as well as the SAR per vessel [data not shown]. But, the electrodes also enable a more uniform exposure of the cell layer. This is proved by a smaller standard deviation and ratio of maximum and minimum field strength in the cell layer compared to results of the vessel without electrodes.

Thermal response of the exposure setup

In order to verify athermal conditions during exposure experiments, a threshold for maximum RF input power had to be determined, because absorbed RF-energy leads to a temperature increase of the cell culture medium within the vessels. For this purpose the temperature increase at the location of the cell layer was measured as function of the input power by use of a fiber optic probe which was plunged through a bore in the metallic cover into the vessel. Results in Fig. 5 show that the scheduled value of 37 °C, controlled by the incubator to be within a margin of ± 0.5 °C, was maintained for a maximum input power of 1.5 W. The corresponding mean value of the electric field across the cell layer is 40 V/m with a standard deviation of 50%. The corresponding SAR is 3.5 W/kg at a vessel volume of 3.5 mL. Two lower exposure levels were selected by stepping two decades back to 0.35 W/kg (10.8 V/m) and 0.035 W/kg (3.4 V/m).

Thermal stress response of PBEC

PBEC showed limited tolerance to highly elevated temperatures as indicated by the enhanced permeability for sucrose (Fig. 6). Temperature elevation of 1K to 38°C almost doubled sucrose permeation compared to the control group within 3 days. The same was observed already after 1 day at 39°C, whereas sucrose permeability was increased by a factor of 5 after 3 days at that temperature. However, the maximum temperature increase of +0.3 K observed at the maximum RF dose of 34 V/m did not change sucrose permeability over the observation period of 3 days.

Transendothelial electrical resistance

Impedance spectra were recorded during 84 h exposure of endothelial cells at different fields. For all exposure conditions, including sham-exposure, we observed an increase in TEER reaching a maximum of approx. 120% of the initial value after 15-20 h. Following this maximum in barrier tightness, the TEER declined almost linearly down to 50 % at the end of every experiment. At this point the absolute TEER value of the cultures was already below 100 $\Omega\cdot\text{cm}^2$ indicating a loss of proper barrier function, thus the recording was terminated. Fig. 7 shows the results of TEER measurements. For better comparison of results obtained with different cell batches, TEER was normalized and given as percent of the initial value. The exposure of PBEC to UMTS-fields from 3.4-34 V/m did not affect the course of TEER development within the period of observation. TEER of exposed PBEC did not differ from that of sham exposed cells at any time. An impact of the applied radiation on the tightness of the BBB in vitro-model, expressed by the electrical resistance across the cell monolayer, could therefore be excluded. Heating of the incubator to 45°C served as positive control as shown in Fig. 8. and led to a rapid breakdown of BBB function.

Permeation experiments

The barrier integrity and function was further investigated by determination of permeation coefficients of several compounds serving as marker molecules, which were divided into two groups. ^{14}C -sucrose is a very small molecule showing a restricted passage across the cell layer, ^{125}I -BSA (bovine serum albumin) is a larger marker molecule which is, besides sucrose, commonly used to quantify BBB tightness. D-glucose, L-alanin and L-leucin (all of them ^3H -labelled) are nutrients that are transported across the BBB and thus were selected for monitoring the influence of EMF on BBB-specific carrier

systems. Permeation coefficients were determined at exposure times of 1-3 days for all markers and at three exposure levels.

The apparent permeation of sucrose ($1.4 \cdot 10^{-6}$ cm/s) and BSA ($2.8 \cdot 10^{-7}$ cm/s) in sham-exposed PBEC cultures indicates a high barrier tightness, being a prerequisite for the investigation of EMF-related influence on BBB properties. A functional expression of intact transporters is indicated by the enhanced permeation of glucose ($6.7 \cdot 10^{-6}$ cm/s), alanin ($5.5 \cdot 10^{-6}$ cm/s) and leucin ($4.9 \cdot 10^{-6}$ cm/s). For better comparison of the results obtained from either exposed or sham exposed cells, the ratio of permeation coefficients (P) for each exposure time and each field strength was calculated as $P_{\text{exposed}}/P_{\text{sham}}$. A permeation ratio >1 therefore indicates an increase in permeability of the BBB.

Fig. 9 shows the permeation ratio of the two markers selected to analyze barrier tightness, sucrose and BSA. Two positive controls are included to quantify the degree of change in barrier tightness in case a severe disruption is induced by means of thermal damage or the influence of a barrier-opening chemical. No change in BBB tightness for sucrose or BSA was observed following EMF exposure at all doses and times. Maximal deviations of a permeation ratio of 1 were observed for BSA after exposure at 10.8 V/m. But at values of 0.78 after one day exposure and 1.28 after two day exposure they were distinctly below a range of variation that must be expected for changes in barrier tightness which would be of biological significance. Examples for a clear BBB-disruption are depicted by the positive controls. Elevation of the incubator temperature to 45°C led to a permeation ratio of 104 for BSA and 34 for sucrose. After the application of a 1.4 M mannitol solution the permeation ratio was 88 for BSA and 44 for sucrose.

We further did not observe any effect of the applied UMTS-radiation on the transport of glucose, leucin an alanin at the BBB in vitro (Fig. 10). During exposure for up to three days at field strengths as described above, the permeation ratio of alanin was in a range of 0.79-1.41, leucin shows a permeation ratio of 0.80-1.09 and glucose of 0.79-1.18. Since all ratios are as well within the standard deviation and variations are observed in both directions, these data do not point to any influence of UMTS radiation on the transport of these nutrients across the BBB in vitro.

Immunocytochemistry

In order to visualize a possible influence of EMF-exposure on tight junction morphology, immunocytochemical stainings of occludin and ZO-1 were conducted after exposure times of 1-3 days for three exposure levels. Fig. 11 and 12 show a comparison between fluorescence micrographs of the TJ proteins after exposure and sham-exposure of PBEC. All pictures show the typical distribution of both proteins at the cell periphery, outlining the spindle-shaped appearance of microvascular endothelial cells in vitro. None of the exposure conditions selected induced visible changes in the staining pattern of occludin or ZO-1. The signal did neither weaken nor change its localization or distribution around the cell borders. A slightly fuzzy appearance in some of the immunostainings of TJ proteins is visible at maximal exposure time. As the same effect can be observed in sham-exposed samples this must not be attributed to field effects but to beginning decrease in barrier stability due to senescence of the cultures.

Western Blot Analysis

The results of occludin and ZO-1 immunostainings were confirmed by Western Blot analysis of PBEC culture lysates. An exposure time of 3 d was selected and the field was set to exposure levels of either 34, 10.8 and 3.4 V/m. As depicted in Fig. 13, occludin features a relatively broad band at a molecular weight of approx. 65 kDa which is attributed to different states of phosphorylation. ZO-1 (MW ~ 225 kDa) shows a typical double band in western blot analysis representing two alternate splicing variants (α^+ and α^-). UMTS exposure did not induce changes in the expression level of ZO-1 and occludin. Bands of both TJ proteins remained identical to those of sham-exposed cells concerning their staining pattern as well as the intensity and retention in SDS-PAGE at all exposure levels.

Discussion

In the present study we investigated the influence of electromagnetic fields as emitted by mobile phones according to the UMTS standard on the integrity and function of the blood-brain barrier. Primary cultures of PBEC served as an *in vitro* model of the BBB. Our investigations were focused on several parameters that are important for proper function of the BBB. Barrier tightness was quantified by means of both TEER and the permeation of sucrose and serum albumin across the cellular monolayer. Transport processes at the BBB were assessed using permeation assays with substrates of different transporters at the BBB: glucose, alanine and leucine. We further analyzed the influence of EMF exposure on cellular proteins that play a central role in the formation of tight junctions. The localization of occludin and ZO-1 at the cellular borders was visualized by immunocytochemistry. Expression level and integrity of these TJ-proteins were characterized by western-blot analysis.

EMF exposure at three different doses (3.4 V/m, 10.8 V/m, 34 V/m) was conducted for up to 3 days. This is the maximal period of time during which primary PBEC display a high barrier tightness *in vitro*. In order to simulate a worst case scenario we chose a permanent exposure for our experiments. For the same reason exposure doses were set to a maximal level without evoking thermal damage. Given a volume of 3.5 mL of culture medium in which the cell-covered filter is located during exposure, the SAR at a dose 34 V/m was calculated as 1.8 W/kg. This value covers the upper limits set by safety guidelines for maximum emission of mobile phones. EMF exposure of PBEC was accompanied by a continuous monitoring of the TEER. Permeation experiments were carried out daily. Immunostainings for occludin and ZO-1 were performed after 24 h and 72 h exposure and western blots were made from cell lysates after 72 h exposure.

As reported here, we did not observe any EMF-related effect on the BBB *in vitro*. Barrier tightness as a most important feature of a functional BBB was assayed by permeation experiments with BBB-impermeable markers of different molecular size, sucrose (MW ~ 0.4 kDa) and BSA (MW ~ 67 kDa). Permeation coefficients of both indicated a high barrier tightness and did not increase due to EMF exposure at any time or any field strength. This is in contradiction to effects that were reported by Salford (Salford et al., 2003). The Swedish group observed serum albumin extravasation in rat brains

after exposure to a GSM 900 signal at 2-200 mW/kg, but did not quantify the extent of albumin leakage.

In our experiments, the TEER as additional indicator of barrier integrity, also remained unaffected during the entire exposure period. In contrast to other techniques, impedance analysis for determination of the TEER allows for continuous monitoring of barrier tightness during RF exposure. Due to this “online” monitoring, we can exclude that potential damage which might have occurred during exposure is disguised by a rapid recovery of the cells after removing them from the waveguide. The method is non-invasive and cell cultures remain undisturbed in the closed incubator during the entire experiment. A frequency scan at a single filter insert takes ~ 6 min and only one filter is measured at a time. Any irritation of the BBB cultures by repeated application of the impedance signal can therefore be excluded. Given a set of e.g. 40 samples, the impedance signal applied to a particular filter only once in a 4 hours period for a very short time only. Unchanged integrity of the barrier as it was found looking at the permeation of marker molecules was confirmed by the stability of the TEER during exposure.

Thermal and hyperosmolar barrier opening served as positive control experiments. They clearly demonstrate the order of magnitude in permeability change if the barrier was disrupted. Sucrose permeation increased by a factor of 34 due to thermal damage and 44-fold after hyperosmolar barrier opening. BSA permeability increased 104-fold and 88-fold, respectively. Marginal variations of TEER and permeation as they were observed under EMF-exposure can therefore definitely be considered as background noise.

Consistently, unchanged barrier properties were also indicated by the integrity of the TJ proteins occludin and ZO-1 that are described to play a central role in TJ formation. They were tested for changes in immunofluorescence and western blot staining patterns that might indicate a loss in barrier integrity. For example, Lohmann (Lohmann et al., 2004) report BBB disruption, utilizing the same in vitro model as used in the present study, induced by the tyrosine phosphatase inhibitor phenylarsine oxide. A loss of barrier tightness was accompanied by occludin proteolysis which became clearly evident in immunocytochemical staining as well as in western blot analyses (Lohmann et al., 2004). Moreover, barrier breakdown was paralleled by a profound disruption of cell-cell contacts as shown in

immunocytochemical stainings of occludin and ZO-1. In our experiments we neither observed a disassembly of cellular contacts in immunofluorescence micrographs nor a cleavage of occludin or ZO-1 in western blots, indicating a lack of influence of EMF on the integrity of these TJ proteins. In addition to the unaffected barrier tightness indicated by unchanged TEER and marker permeation, these findings further confirm our conclusion that the generic UMTS field applied did not induce changes in morphology nor function of the BBB in vitro.

Nevertheless TJ consist of a very complex system of protein and membrane components. For review see: (D'Atri and Citi, 2002; Wegener and Galla, 1996) and their precise composition is not yet clarified. A large number of proteins are involved in TJ formation, thus the stability of occludin and ZO-1 does not exclude the possibility that other TJ proteins might be affected by electromagnetic fields as applied here. A screening process on a molecular level would be necessary to gain further insights into the expression and regulation of TJ components upon EMF exposure.

Risk assessment of potential EMF hazards has gained broad public interest in recent years and research results are often misinterpreted by reduction of complex experimental data to a simple 'yes' or 'no' answer. We therefore think that it is important point out that the present study cannot give final answers to the question whether or not EMF emission by mobiles phones generally is harmless to humans. EMF risk assessment covers a wide range of biological targets to receive potential damage as well as a broad spectrum of EMF frequencies and their modulations. Our study puts a focus on fundamental parameters of BBB function upon exposure to UMTS-like mobile phone signals. As the nature of the BBB is very complex and not yet entirely understood, it is impossible to address all BBB-related aspects in one approach.

The use of an vitro model of the BBB with cells from porcine origin raises the question about portability of results between species. It is evident that we cannot directly extrapolate from these results to health hazards for humans. However, this was not the aim of our study. Instead, basic research as conducted in our study aims to elucidate the discussion on possible molecular targets and interactions of biological tissue components with EMF. For this purpose it is exceptionally helpful to introduce distinct reductions to the complexity of biological systems. This issue is solved well by an in

vitro system comprising only one single type of cells, PBEC in our case. These cell cultures of brain microvascular endothelium present an alternative and powerful method to assay BBB tightness. A major advantage of this approach is the elimination of mental stress which is likely to be evoked by restraining of test animals during exposure. Even unrestrained animals may be stressed by repeated transfers into exposure cages. Our exposure unit was designed to meet the typical requirements of the cell cultures, for example, it was installed completely within a standard incubator. Cell cultures permit precise field calculations as their position within the exposure unit is permanently fixed, in contrast to freely moving animals of different size and weight. In order to investigate athermal effects, cell cultures facilitate temperature control as a temperature probe can easily be added directly into the test system.

So far, only one other study deals with the investigation of EMF induced changes in BBB-integrity in vitro. Schirmacher (Schirmacher et al., 2000) reported a barrier disrupting impact of EMF (GSM-1800) on an in vitro model similar to the one we used in the present study. They observed a two-fold increase in sucrose permeation after four days exposure at 0.3 W/kg. After optimizing culture conditions to receive a high, close to in vivo barrier tightness as we report here, we failed to reproduce their observations with the identical exposure setup (data submitted for publication).

To conclude, we showed that a generic UMTS-field did not cause adverse effects to an in vitro model of the BBB at subthermal exposure conditions. Barrier tightness, assessed by marker permeation and electrical resistance (TEER), transport behavior of selected compounds and the integrity and distribution of TJ proteins occludin and ZO-1 remained unchanged after 1-3 d exposure at 3.4-34 V/m (0.02-1.8 W/kg). Since the TEER was monitored simultaneously during the exposure process, any effect which might be detectable only in presence of the field is not feasible either.

Due to these findings, it is likely that EMF from mobile phones of the UMTS generation do not substantially harm the blood-brain barrier. Further experiments are ongoing and will focus on investigations of differential gene expression determined by chip array systems. This will provide a basis for the identification of biological molecular targets that might interact with electromagnetic fields.

Acknowledgements: We thankfully acknowledge financial support of this study by Forschungsgemeinschaft Funk e.V. – Research Association for Radio Applications (Contract No. 5610). We thank Heiner Nattkämper for his cooperation during his diploma thesis.

Figure legends:

Fig. 1: Scheme of the exposure system for investigations of an in vitro model of the BBB.

Fig. 2 : Vessel, filter insert and electrodes.

Fig. 3 Mounted exposure and measuring unit and its installation in the waveguide.

Fig. 4 Distribution of the electrical field strength across the cell layer.

Fig. 5. Temperature increase in the culture medium due to UMTS exposure versus average electrical field strength across the cell layer. The arrow for the highest field tested (102.2 V/m; 22.65 W/kg) indicates a further increasing of the temperature with time at this point.

Fig. 6 Sucrose permeation ratio across PBEC at elevated temperatures. No change is observed at 37°C (control experiment) and 37.3°C (condition at 34 V/m exposure). Moderate elevation of permeability can be seen at 38°C whereas at 39°C, after 72h the barrier is severely damaged. Values given as mean \pm SD (n= 6).

Fig. 7 TEER during EMF-exposure at different exposure levels. Open circles: sham exposed; black triangles: exposed. Data are given as mean \pm SD (n=25-35).

Fig 8. Positive control: Temperature was elevated to 45°C at 1500 min. Data are given as mean \pm SD (n=6).

Fig. 9 Permeation ratio of sucrose and BSA. Cells were exposed at different levels for 1-3 d. Two positive controls quantify the degree of change in barrier tightness after thermal and hyperosmolar barrier opening. Data are given as mean \pm SD. (BSA: n=4; sucrose: n \geq 10)

Fig 10. Permeation ratio of alanin, leucin and glucose. Cells were exposed at different levels for 1-3 d. Data are given as mean \pm SD (n=4).

Fig 11. Fluorescence micrographs of ZO-1 immunostaining after 24 or 72 h exposure to UMTS-EMF at three different field strengths and sham exposure. Red: ZO-1 staining, blue: DAPI staining of nuclei.

Fig 12. Fluorescence micrographs of occludin immunostaining after 24 or 72 h exposure to UMTS-EMF at three different field strengths and sham exposure. Green: occludin staining, blue: DAPI staining of nuclei.

Fig. 13. Immunoblot analysis of tight junction proteins after EMF-exposure. PBEC were exposed to levels of 3.4 V/m, 10.8 V/m and 34 V/m for 3d.

References

- Ando-Akatsuka Y, Saitou M, Hirase T, Kishi M, Sakakibara A, Itoh M, et al. Interspecies diversity of the occludin sequence: cDNA cloning of human, mouse, dog, and rat-kangaroo homologues. *J Cell Biol* 1996; 133: 43-7.
- Balda MS, Anderson JM. Two classes of tight junctions are revealed by ZO-1 isoforms. *Am J Physiol* 1993; 264: C918-24.
- Butt AM, Jones HC, Abbott NJ. Electrical resistance across the blood-brain barrier in anaesthetized rats: a developmental study. *J Physiol* 1990; 429: 47-62.
- Crone C. Facilitated transfer of glucose from blood into brain tissue. *J Physiol* 1965; 181: 103-13.
- D'Atri F, Citi S. Molecular complexity of vertebrate tight junctions (Review). *Mol Membr Biol* 2002; 19: 103-12.
- Engelbertz C, Korte D, Nitz T, Franke H, Haselbach M, Galla H-J. The development of in vitro models for the blood-brain- and the blood-CSF-barrier In: *Blood-brain-barrier and drug delivery to the CNS*. W. Kreuter, D. Begley and M. Bradbury, eds. Marcel Dekker Inc. New York 2000: 33-63.
- Finnie JW, Blumbergs PC, Manavis J, Utteridge TD, Gebski V, Davies RA, et al. Effect of long-term mobile communication microwave exposure on vascular permeability in mouse brain. *Pathology* 2002; 34: 344-7.
- Finnie JW, Blumbergs PC, Manavis J, Utteridge TD, Gebski V, Swift JG, et al. Effect of global system for mobile communication (gsm)-like radiofrequency fields on vascular permeability in mouse brain. *Pathology* 2001; 33: 338-40.
- Franke H, Galla H-J, Beuckmann CT. An improved low-permeability in vitro-model of the blood-brain barrier: transport studies on retinoids, sucrose, haloperidol, caffeine and mannitol. *Brain Res* 1999; 818: 65-71.
- Franke H, Galla H-J, Beuckmann CT. Primary cultures of brain microvessel endothelial cells: a valid and flexible model to study drug transport through the blood-brain barrier in vitro. *Brain Res Brain Res Protoc* 2000; 5: 248-56.

- Fritze K, Sommer C, Schmitz B, Mies G, Hossmann KA, Kiessling M, et al. Effect of global system for mobile communication (GSM) microwave exposure on blood-brain barrier permeability in rat. *Acta Neuropathol (Berl)* 1997; 94: 465-70.
- Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S. Occludin: a novel integral membrane protein localizing at tight junctions. *J Cell Biol* 1993; 123: 1777-88.
- Furuse M, Itoh M, Hirase T, Nagafuchi A, Yonemura S, Tsukita S. Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. *J Cell Biol* 1994; 127: 1617-26.
- Furuse M, Sasaki H, Fujimoto K, Tsukita S. A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. *J Cell Biol* 1998; 143: 391-401.
- Goldstein GW, Betz AL. The blood-brain barrier. *Sci Am* 1986; 255: 74-83.
- Haskins J, Gu L, Wittchen ES, Hibbard J, Stevenson BR. ZO-3, a novel member of the MAGUK protein family found at the tight junction, interacts with ZO-1 and occludin. *J Cell Biol* 1998; 141: 199-208.
- Hirase T, Staddon JM, Saitou M, Ando-Akatsuka Y, Itoh M, Furuse M, et al. Occludin as a possible determinant of tight junction permeability in endothelial cells. *J Cell Sci* 1997; 110 (Pt 14): 1603-13.
- Hoheisel D, Nitz T, Franke H, Wegener J, Hakvoort A, Tilling T, et al. Hydrocortisone reinforces the blood-brain properties in a serum free cell culture system. *Biochem Biophys Res Commun* 1998; 247: 312-5.
- Itoh M, Furuse M, Morita K, Kubota K, Saitou M, Tsukita S. Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins. *J Cell Biol* 1999a; 147: 1351-63.
- Itoh M, Morita K, Tsukita S. Characterization of ZO-2 as a MAGUK family member associated with tight as well as adherens junctions with a binding affinity to occludin and alpha catenin. *J Biol Chem* 1999b; 274: 5981-6.

- Jesaitis LA, Goodenough DA. Molecular characterization and tissue distribution of ZO-2, a tight junction protein homologous to ZO-1 and the *Drosophila* discs-large tumor suppressor protein. *J Cell Biol* 1994; 124: 949-61.
- Keese CR, Bhawe K, Wegener J, Giaever I. Real-time impedance assay to follow the invasive activities of metastatic cells in culture. *Biotechniques* 2002; 33: 842-4, 846, 848-50.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-5.
- Levin VA. Relationship of octanol/water partition coefficient and molecular weight to rat brain capillary permeability. *J Med Chem* 1980; 23: 682-4.
- Lohmann C, Huwel S, Galla H-J. Predicting blood-brain barrier permeability of drugs: evaluation of different in vitro assays. *J Drug Target* 2002; 10: 263-76.
- Lohmann C, Krischke M, Wegener J, Galla H-J. Tyrosine phosphatase inhibition induces loss of blood-brain barrier integrity by matrix metalloproteinase-dependent and -independent pathways. *Brain Res* 2004; 995: 184-96.
- Ndoubè Mbonjo Mbonjo H, Streckert J, Bitz A, Hansen V, Glasmachers A, Gencol S, et al. A generic UMTS test signal for RF bio-electromagnetic studies. *Bioelectromagnetics* 2004; accepted for publication.
- Persson BR, Salford LG, Brun A. Blood-brain barrier permeability in rats exposed to electromagnetic fields used in wireless communication. *Wireless Networks* 1997; 3: 455-461.
- Salford LG, Brun A, Stureson K, Eberhardt JL, Persson BR. Permeability of the blood-brain barrier induced by 915 MHz electromagnetic radiation, continuous wave and modulated at 8, 16, 50, and 200 Hz. *Microsc Res Tech* 1994; 27: 535-42.
- Salford LG, Brun AE, Eberhardt JL, Malmgren L, Persson BR. Nerve cell damage in mammalian brain after exposure to microwaves from GSM mobile phones. *Environ Health Perspect* 2003; 111: 881-3; discussion A408.
- Schirmacher A, Winters S, Fischer S, Goeke J, Galla H-J, Kullnick U, et al. Electromagnetic fields (1.8 GHz) increase the permeability to sucrose of the blood-brain barrier in vitro. *Bioelectromagnetics* 2000; 21: 338-45.

- Schneeberger EE, Lynch RD. Structure, function, and regulation of cellular tight junctions. *Am J Physiol* 1992; 262: L647-61.
- Stevenson BR, Siliciano JD, Mooseker MS, Goodenough DA. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J Cell Biol* 1986; 103: 755-66.
- Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. *Nat Rev Mol Cell Biol* 2001; 2: 285-93.
- Tsurita G, Nagawa H, Ueno S, Watanabe S, Taki M. Biological and morphological effects on the brain after exposure of rats to a 1439 MHz TDMA field. *Bioelectromagnetics* 2000; 21: 364-71.
- Wegener J, Galla H-J. The role of non-lamellar lipid structures in the formation of tight junctions. *Chem Phys Lipids* 1996; 81: 229-255.
- Wegener J, Hakvoort A, Galla H-J. Barrier function of porcine choroid plexus epithelial cells is modulated by cAMP-dependent pathways in vitro. *Brain Res* 2000; 853: 115-24.
- Wegener J, Sieber M, Galla H-J. Impedance analysis of epithelial and endothelial cell monolayers cultured on gold surfaces. *J Biochem Biophys Methods* 1996; 32: 151-70.