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Increased blood–brain barrier permeability in mammalian brain 7 days after exposure to the radiation from a GSM-900 mobile phone

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Abstract

Microwaves were for the first time produced by humans in 1886 when radio waves were broadcasted and received. Until then microwaves had only existed as a part of the cosmic background radiation since the birth of universe. By the following utilization of microwaves in telegraph communication, radars, television and above all, in the modern mobile phone technology, mankind is today exposed to microwaves at a level up to 10^{20} times the original background radiation since the birth of universe.

Our group has earlier shown that the electromagnetic radiation emitted by mobile phones alters the permeability of the blood–brain barrier (BBB), resulting in albumin extravasation immediately and 14 days after 2 h of exposure.

In the background section of this report, we present a thorough review of the literature on the demonstrated effects (or lack of effects) of microwave exposure upon the BBB.

Furthermore, we have continued our own studies by investigating the effects of GSM mobile phone radiation upon the blood–brain barrier permeability of rats 7 days after one occasion of 2 h of exposure. Forty-eight rats were exposed in TEM-cells for 2 h at non-thermal specific absorption rates (SARs) of 0 mW/kg, 0.12 mW/kg, 1.2 mW/kg, 12 mW/kg and 120 mW/kg. Albumin extravasation over the BBB, neuronal albumin uptake and neuronal damage were assessed.

Albumin extravasation was enhanced in the mobile phone exposed rats as compared to sham controls after this 7-day recovery period (Fisher's exact probability test, $p = 0.04$ and Kruskal–Wallis, $p = 0.012$), at the SAR-value of 12 mW/kg (Mann–Whitney, $p = 0.007$) and with a trend of increased albumin extravasation also at the SAR-values of 0.12 mW/kg and 120 mW/kg. There was a low, but significant correlation between the exposure level (SAR-value) and occurrence of focal albumin extravasation ($r_s = 0.33$; $p = 0.04$).

The present findings are in agreement with our earlier studies where we have seen increased BBB permeability immediately and 14 days after exposure. We here discuss the present findings as well as the previous results of altered BBB permeability from our and other laboratories.

Keywords: Albumin; Blood–brain barrier; Mobile phone; Rat

1. Introduction: radiofrequency radiation and the blood–brain barrier

Today about half of the world's population owns the microwave-producing mobile phones. An even larger number is exposed to the radiation emitted from these devices through "passive mobile phoning" [1]. Life-long exposure to the microwaves (MWs) from mobile phones, with start already at a young age, is becoming increasingly common

Abbreviations: BBB, blood–brain barrier; CNS, central nervous system; CW, continuous wave; EMF, electromagnetic field; GSM, global system for mobile communication; ICNIRP, International Commission of Non-ionizing Radiation Protection; MRI, magnetic resonance imaging; RF, radio frequency; SAR, specific absorption rate; TEM-cell, transverse electromagnetic transmission line chamber.

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among the new generations of mobile phone users. The question is: to what extent are living organisms affected by these radio frequency (RF) fields?

The mobile phones are held in close proximity to the head, or within a metre of the head when hands-free sets are used. The emitted microwaves have been shown to have many effects upon the mammalian brain; e.g. alterations of cognitive functions [2,3], changes of neurotransmitter levels such as decrease of cholinergic activity [4], gene expression alterations in cerebellum [5], cortex and hippocampus [6], and impact upon the brain EEG activity [7]. Also, the human brain EEG beta rhythms energies were increased by exposure to 450 MHz MWs modulated at different low frequencies [8]. Recent epidemiological studies also indicate that long-term exposure increases the risk of not only for benign vestibular schwannoma (previously named acoustic neurinoma) [9], but also malignant glioblastoma multiforme [10] for mobile phone use longer than 10 years and with cumulative exposure from mobile phones exceeding 2000 h.

It has been shown that electromagnetic fields (EMFs) increase the permeability of the blood–brain barrier (BBB) (for reference see [11]). The BBB is a hydrophobic barrier, formed by vascular endothelial cells of the capillaries in the brain, with tight junctions between these endothelial cells. It protects the mammalian brain from potentially harmful compounds in the blood. Also, perivascular structures such as astrocytes and pericytes as well as a bi-layered basal membrane help maintaining the BBB.

The current recommendations for limits of exposure to the general public for EMF radiation [12] are set in order to avoid thermal effects upon the brain parenchyma.

In our previous studies we have seen that non-thermal RF fields cause significantly increased leakage of the rats' own albumin through the BBB of exposed rats sacrificed immediately after the exposure, as compared to sham exposed control animals [11,13–18]. Two hours of exposure to the radiation from a global system for mobile communications (GSM) phone at 915 MHz, at non-thermal specific absorption rates (SAR) values of 0.12 mW/kg, 12 mW/kg and 120 mW/kg, gives rise to focal albumin extravasation and albumin uptake into neurons also 14 days after exposure, but not after 28 days [19]. Significant neuronal damage is present 28 days [19] and 50 days after exposure [20], but not after 14 days [19]. Also, in experiments from other laboratories, BBB permeability is increased in connection to mobile phone exposure [21–23] and other kinds of EMF such as magnetic resonance imaging (MRI) exposure [24–26]. In other studies, no such BBB alterations have been demonstrated in connection to mobile phone exposure [27–29] or other kinds of EMF exposure [30,31].

1.1. The blood–brain barrier

An intact BBB is necessary for the protection of the mammalian brain from potentially harmful substances circulating in the blood. In the normal brain, the passage of compounds over the BBB is highly restricted and homeostasis within the sensitive environment of the brain parenchyma can be maintained.

The BBB is formed by the vascular endothelial cells of the capillaries of the brain and the glial cells wrapped around them. The tight junctions, that seal the endothelial cells together, limit paracellular leakage of molecules. A bi-layered basal membrane supports the ablumenal side of the endothelial cells. The glial astrocytes, surrounding the surface of the basal membrane cells, are important for the maintenance, functional regulation and repair of the BBB. The protrusions of the astrocytes, called end feet, cover the basal membrane on the outer endothelial surface and thus form a second barrier to hydrophilic molecules and connect the endothelium to the neurons. Twenty-five per cent of the ablumenal membrane of the capillary surface is covered by pericytes [32], which are a type of macrophages. Seemingly, they are in the position to significantly contribute to the central nervous system (CNS) immune mechanisms [33].

The physiological properties of the CNS microvasculature are different from those of peripheral organs. The numbers of pinocytotic vesicles for nutrient transport through the endothelial cytoplasm are low and there are no fenestrations. Also, there is a fivefold higher number of mitochondria in the BBB endothelial cells as compared to muscular endothelial cells [34].

In a functioning BBB, the membrane properties control the bidirectional exchange between the general circulation and the CNS. Water, most lipid-soluble molecules, oxygen and carbon dioxide can diffuse from the blood to the nerve cells. The barrier is slightly permeable to ions such as sodium, potassium and chloride, but large molecules, such as proteins and most water-soluble chemicals only pass poorly. However, when this barrier is damaged, in conditions such as tumours, infarcts or infections, also the normally excluded molecules can pass through, possibly bringing toxic molecules out into the brain tissue. The selective permeability is disrupted temporally in cases of epileptic seizures [35,36] and severe hypertension [37]. The result of this can be cerebral oedema, increased intracranial pressure and irreversible brain damage. Also, toxic substances from the blood circulation now reach out to the neurons. Even transient openings of the BBB can lead to permanent tissue damage [37].

1.2. The earliest studies on the effects of microwave exposure

The first studies on the MW effects upon the BBB were reported in the 1970s, when the radiation from radars and MW ovens were considered to be possible health threats. Increased leakage of fluorescein after 30 min of pulsed and CW exposure [38] and passage of ^{14}C-mannitol, inulin and dextran at very low energy levels [39] were reported. The permeation of mannitol was found to be a definite function of exposure parameters such as power density, pulse width, and the number of pulses per second. Also, the BBB permeability depended on the time between the EMF exposure and the

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sacrifice of the animals, with more pronounced effects seen in the animals sacrificed earlier after the EMF exposure. In attempts to replicate the findings of Oscar and Hawkins [39], however, these results were not found [40,41]. Similar lack of MW induced BBB effects, was reported by Ward et al. [42] after exposure of rats to CWs at 2450 MHz; Ward and Ali [43] after exposure at 1.7 GHz; and Gruenau et al. [44] after exposure to pulsed or CW waves at 1.8 GHz (including totally 31 rats). On the other hand, Albert and Kerns [45] observed EMF-induced BBB permeability after exposure at 2450 MHz CWs, with an increase in the number of pinocytotic vesicles among the irradiated animals, but after a recovery time of 1–2 h, the permeation was hardly detectable anymore. For details concerning the EMF exposure parameters in these studies, see [11].

1.3. MRI exposure

MRI entails a concurrent exposure to a high-intensity static field, a RF field and a time-varying magnetic field. In connection to the introduction of the MRI technique, the effects of exposure to these kinds of fields upon the BBB permeability were investigated.

As mentioned above, Shivers et al. [24] observed that the EMF exposure of the type emitted during a MRI procedure resulted in a temporarily increased BBB permeability in the brains of rats. Through transendothelial channels, a vesicle-mediated transport of horseradish peroxidase (HRP) took place. Replications of the initial findings by Shivers et al. [24] were made by Garber et al. [46], whereas Adzamli et al. [30] and Preston et al. [31] could not repeat the findings.

After some years, quantitative support of the findings by Shivers et al. [24] was presented by the same group [25,26]. In rats exposed to the MRI, the BBB permeability to DTPA (diethylenetriameninepentaacetic acid) increased. A suggested mechanism explaining the increased permeability was a stimulation of endocytosis, made possible through the time-varying magnetic fields.

Also our studies supported the findings of the Shiver–Prato group; seeing that BBB permeability to albumin was increased after exposure to MRI radiation [13]. The most significant effect was observed after exposure to the RF part of the MRI.

1.4. Studies on mobile phone exposure

The mobile phone induced effects upon the BBB permeability is a topic of importance for the whole society today. We have previously found an increased BBB permeability immediately after 2 h of mobile phone exposure [14], and also after 14 days [19] and 50 days [20].

Repetitions of our findings of increased BBB permeability after mobile phone exposure have been made [47,21,22]. Four hours of GSM-900 MHz exposure at brain power densities ranging from 0.3 to 7.5 W/kg resulted in significantly increased albumin extravasation both at the SAR-value of 7.5 W/kg, which is a thermal effect, but also at 0.3 W/kg and 1.3 W/kg [47] (statistical evaluations discussed by Salford et al. [1]). Albumin extravasation was also seen in rats exposed for 2 h to GSM-900 MHz at non-thermal SAR-values of 0.12, 0.5 and 2 W/kg using fluorescein-labelled proteins [21,22]. At SAR of 2 W/kg a marked BBB permeabilization was observed, but also at the lower SAR-value of 0.5 W/kg, permeabilization was present around intracranial blood vessels. However, the extravasation at 0.5 W/kg was seen at a lesser extent as compared to that seen at 2 W/kg. Subgroups of the rats included in these studies were sympathectomised, which means that they were in a chronic inflammation-prone state with increased BBB opening due to changes in the structures of the blood vessels. Interestingly, the sympathectomised rats exposed to GSM radiation had a remarkable increase of the BBB leakage as compared to their sympathectomised sham controls. From these findings it seems likely that an already disrupted BBB is more sensitive to the RF fields than an intact BBB.

In another study, the uptake of rhodamine–ferritin complex through the BBB was investigated [23]. In this study, increased BBB permeability was clearly seen at exposure levels of 2 W/kg and durations of 30–120 min. When the rats were pre-treated with colchicine, the EMF-induced rhodamine–ferritin uptake was however blocked. Colchicine is well-known for its inhibition of microtubular function. It was concluded that the microtubules seemed to play an important role for the BBB opening.

Lack of EMF-induced BBB alterations has also been reported [27–29,48]. In a small study including only 12 EMF exposed animals, no albumin extravasation was seen, neither after 2 nor 4 weeks of 1 h of daily exposure (average whole-body exposure at 0.25 W/kg) [27]. In a study including 40 animals, Kuribayashi et al. [28] concluded no BBB alterations was seen after 90 min of daily EMF exposure for 1–2 weeks at SAR-values of 2 or 6 W/kg. Finnie et al. [29] exposed mice for 1 h daily. However, only the SAR-value of 4 W/kg, which is above the ICNIRP limit [12], was included. In a further study by Finnie et al. [48] 207 mice were exposed for 104 weeks at SAR-values of 0.25–4 W/kg, however without any observable effects upon the BBB permeability. The same group also reported that the immature BBB was insensitive to mobile phone exposure, seen after GSM-900 radiation exposure of pregnant mice from day 1 to day 19 of gestation (SAR of 4 W/kg, exposure for 60 min daily). No increased albumin extravasation was seen in the new-born mice immediately after parturition [49] and the same lack of GSM-900 radiation effects upon the BBB permeability was reported for young rats by Kumlin et al. [50], however, in this case only 12 out of totally 48 exposed rats were analyzed histopathologically. The remaining rats were subject to memory tests, where an improved learning and memory was seen in the EMF exposed rats as compared to the sham controls. Notably, in all these studies, the SAR-values for exposure are relatively high; never including the low SAR-values below 200 mW/kg.

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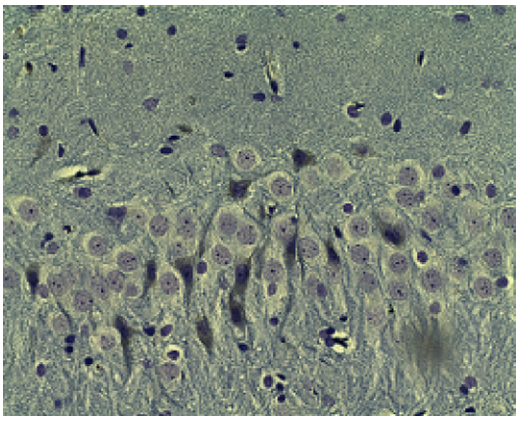

Fig. 1. Albumin neuronal uptake and early neuronopathy in the hippocampal pyramidal cell row among normal neurons. Albumin: cresyl violet, ×20.

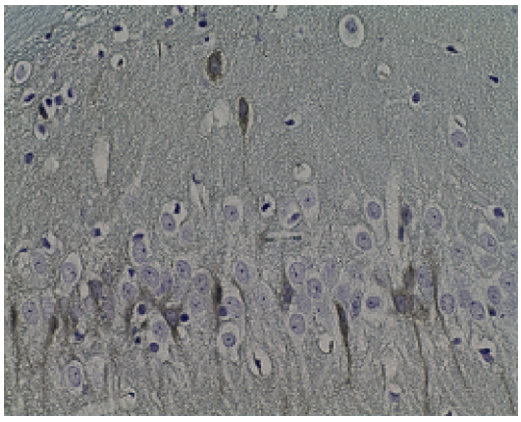

Fig. 2. Shrunken homogenized dark neurons with brownish discoloration due to uptake of albumin, interspersed among normal neurons in the hippocampal pyramidal cell row. Albumin: cresyl violet, ×20. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

In more recent years, *in vitro* models have been increasingly applied to investigate the BBB; in one of these, it was shown that EMFs at 1.8 GHz increase the permeability to sucrose [51]. After modifications of the BBB model to one with higher tightness, however, the same group could not replicate their initial findings [52]. With application of the EMF of the kind emitted from 3G mobile phones, the same group further concluded that their *in vitro* BBB model also did not alter its tightness or transport behaviour in connection to this type of exposure [53].

1.5. Neuronal damage in connection to mobile phone exposure

In our previous studies of animals surviving a longer period after the exposure, we have evaluated the occurrence of neuronal damage extensively [19,20]. This neuronal damage is seen as condensed dark neurons. Dark neurons have been proposed to have three main characteristics [54]: (i) irregular cellular outlines, (ii) increased chromatin density in the nucleus and cytoplasm and (iii) intensely and homogenously stained nucleus. Twenty-eight days after 2 h of mobile phone exposure, the neuronal damage was significantly increased in the exposed rats as compared to the sham exposed controls [19]. Also 50 days after the same kind of mobile phone exposure, there was an increased occurrence of neuronal damage [20].

In our studies, normal neurons have been shown to have increased uptake of albumin [19] (Fig. 1). Also, in dark neurons this neuronal albumin uptake can be seen (Fig. 2). In our previous studies, damaged neurons were seen in all locations, intermingled with normal neurons especially in the cortex, hippocampus and basal ganglia. The damaged neurons were often shrunken and dark staining, homogenized with loss of discernable internal cell structures (Fig. 3). Some damaged neurons showed microvacuoles in the cytoplasm (Fig. 4). These vacuoles are a sign of severe neuronopathy, indicating an active pathological process. There was no evidence of haemorrhages or glial reaction.

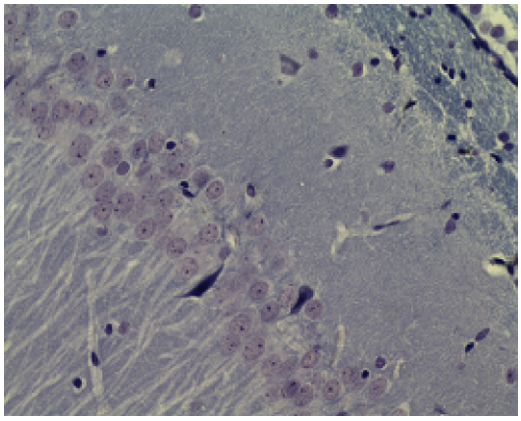

Fig. 3. Two dark neurons in the hippocampal pyramidal cell row. Albumin: cresyl violet, ×20.

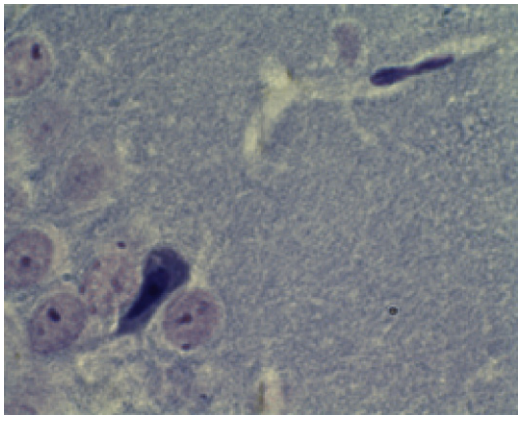

Fig. 4. Dark neuron in the hippocampal pyramidal cell row, with homogenized nucleus and cytoplasm with a vacuole. Higher magnification of part of the figure. Albumin: cresyl violet, ×40.

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Dark neurons are reported in clinical and experimental neuropathology from living tissues, but not in autopsy material unless the post-mortem period is short. This could indicate that the formation of dark neurons is an active process that requires living neurons and that these cells must be reasonably intact [55]. This could be in accordance with our findings from the 50-days survival animals, where apoptosis could not be detected in any of the RF EMF exposed brains with application of Caspase-3 [56].

Dark neurons occur not only after GSM exposure [19,20] but also in connection to experimental ischemia [57], hypoglycemia [58] and epilepsy [59]. Possibly, dark neurons could be artefacts, having a pressure-derived mechanical origin, as has been shown for cortical biopsies (this is less likely considering the atraumatic method of dissection used here including fixation before handling and in view of the deep location of the dark neurons). However, dark neurons also appear as a result of other, and not fully clarified, mechanisms, as seen in the case of GSM exposure, ischemia, hypoglycemia and epilepsy. A pharmacologic origin, such as depolarization related to tissue glutamate release in injury, could explain the pathogenetic mechanism for dark neurons in these cases, rather than the pressure-derived mechanical origin. Indeed, the formation of dark neurons can be prevented using pharmacologic forms of glutamate antagonism [55]. In the case of our studies, our technique for the resection of the rat brains is chosen to avoid mechanical pressure.

Findings of dark neurons in connection to mobile phone exposure have been reported by Ihan et al. [60] (GSM exposure of rats for 7 days, 1 h daily). Also, an increase of oxidative damage was seen in the exposed rats as a significant increase in malondialdehyde (MDA) (an index for lipid peroxidation), nitric oxide (NO) levels, brain xanthine oxidase (XO) and adenosine deaminase (ADA) activities, as compared to the controls. With treatment of the anti-oxidant *Gingko biloba*, the EMF induced increments of XO, ADA, MDA and NO were prevented. The anti-oxidant activity of *G. biloba* is attributed to its flavinoid glycosides, which are the active compounds in the leaves. The action of these flavinoids is to destroy free radicals, such as NO and lipid peroxide radicals. Also the formation of dark neurons was reported to be prevented when the rats had been treated with *G. biloba*. Other attempts to repeat our findings of dark neurons after mobile phone exposure have been performed in a collaborative effort with Bernard Veyret's group in Bordeaux [61]. In this study, the situation 14 days and 50 days after 2 h of GSM-900 radiation exposure at average brain SAR-values of 0.14 W/kg and 2.0 W/kg was evaluated. No increased amount of dark neurons was reported.

It has been suggested that BBB leakage is the major reason for nerve cell injury, such as dark neurons in stroke-prone spontaneously hypertensive rats [62]. Albumin leaks into the brain and neuronal degeneration is seen in areas with BBB disruption in several circumstances: after intracarotid infusion of hyperosmolar solutions in rats [63]; in the stroke prone hypertensive rat [65]; in acute hypertension by aortic compression in rats [37]. The linkage between albumin extravasation over the BBB and neural damage might be a potentiating effect of albumin upon the glutamate-mediated neurotoxicity [64]. Indeed, both albumin- and glutamate-induced lesions have the same histopathological appearance with invasion of macrophages and absence of neuronal cell bodies and axons in the lesion areas [65]. The glutamate itself can also increase the BBB opening [66], leading to further albumin extravasation out into the brain parenchyma. From our previous findings of albumin extravasation 14 days after exposure [19] and dark neurons not until after 28 days [19] and 50 days [20], it could be hypothesized that albumin extravasation into the brain parenchyma, is the first observable effect of the mobile phone exposure. The albumin leakage precedes and possibly could be the cause of, the damage to the neurons seen as the dark neurons later on. In this connection, the findings of [37] that transient openings of the BBB can result in permanent tissue damage, can also be mentioned. Hypertensive opening of the BBB resulted in albumin extravasation after 2 h, but the effects remained, although to a lesser extent, also after 7 days. Many neurons with cytoplasmatic immunoreactivity for albumin appeared shrunken. Seven days after the BBB opening, there was a neuronal loss in these areas and a vigorous glial reaction, indicating that some neurons were irreversibly damaged [37].

2. Aims of the present study

In the present study we have continued to investigate the effects of EMFs upon the rat brain, now with focus on what happens 7 days after GSM exposure at 915 MHz for 2 h at non-thermal energy levels of 0.12 mW/kg, 1.2 mW/kg, 12 mW/kg and 120 mW/kg. The main questions to be answered were: whether the same increase of the BBB permeability is seen 7 days after exposure as that showed previously immediately after exposure and after 14 days, and whether different exposure levels result in a different response.

In order to compare to our previous findings, we have used the same exposure system, GSM signal, animal model and histopathological methods as in our previous studies.

3. Materials and methods

3.1. GSM exposure

The animals were exposed to RF EMFs in the same transverse electromagnetic transmission line cell (TEM-cells) as previously described and used by [1,2,5,13–19]. The TEM-cells were designed by dimensional scaling from previously constructed cells at the National Bureau of Standards [67]. TEM-cells are known to generate uniform EMFs for standard measurements.

A genuine GSM mobile phone, operating at the 900 MHz frequency band, with programmable power output, was con-

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nected via a coaxial cable to the TEM-cells. Through a power splitter, the power was divided into equal parts fed into the four TEM-cells used (TEM-cell A, B, C and D). No voice modulation was applied. Each of the four TEM-cells is connected to a 50 Ω dummy load, into which the output is terminated. By using these TEM-cells, the pulse modulated exposure fields can be accurately generated without the distortion that is typically introduced when conventional antennas are used to establish impulse test fields. Thus, a relatively homogeneous exposure of the animals is allowed [68].

The TEM-cell is enclosed in a wooden box (inner dimensions of 15 cm × 15 cm × 15 cm) that supports the outer conductor, made of brass net, and central conducting plate. The central plate separates the top and bottom of the outer conductor symmetrically. Eighteen holes (diameter 18 mm) in the sidewalls and top of the wooden box make ventilation possible. Air is circulated through the holes of the TEM-cells using four fans, each placed next to the outer walls of its respective TEM-cell. The holes are also used for examination of the interior during exposure. For a further description of the TEM-cell, see [68].

The rats were placed in plastic trays (14 cm × 14 cm × 7 cm) to avoid contact with the central plate and outer conductor. The bottom of the tray was covered with absorbing paper to collect urine and faeces. Each TEM-cell contained two plastic trays, one above and one below the centre septum. Thus two rats could be kept in each TEM-cell simultaneously. All the animals could move and turn around within the TEM-cells.

For the actual experimental situation with one rat in each compartment of the TEM-cell, the conversion factor K for SAR per unit of input power could be fitted to the data as

$$K = (1.39 \pm 0.17) - (0.85 \pm 0.22)w \quad (1)$$

with w the sum of weights in kilograms of the 2 rats in the cell and the variance given as SEM. For a more detailed description, see [2].

Whole-body SAR and brain SAR vary with orientation. In our present set-up, the average of SAR for the brain grey matter was 1.06 times the average whole-body SAR, with a standard deviation of 56% around the average value for the different orientations, as estimated by us previously [19].

3.2. Animals

All animal procedures were performed according to the practices of the Swedish Board of Animal Research and were approved by the Animal Ethics Committee, Lund-Malmö.

Forty-eight inbred male and female Fischer 344 rats (the rats were supplied by Scanbur AB, Stockholm, Sweden) were 2–3 months of age at the initiation of the EMF exposure. Male and female rats weighed 225 g ± 56 g (standard deviation) and 233 g ± 60 g (standard deviation) respectively. The rats were housed in rat hutches, two in each cage, under standard conditions of 22 °C room temperature, artificial daylight illumination and rodent chow and tap water *ad libitum*.

The 48 rats were divided into four exposure groups, each group consisting of 8 rats, and one sham exposed group with 16 animals.

The peak power output from the GSM mobile phone fed into the TEM-cells was 1 mW, 10 mW, 100 mW and 1000 mW per cell respectively for a period of 2 h. This resulted in average whole-body SAR of 0.12 mW/kg, 1.2 mW/kg, 12 mW/kg and 120 mW/kg for the four different exposure groups.

All animals were kept in the animal facilities for a recovery period of 7 days after exposure. At the end of this period they were anaesthetized and sacrificed by perfusion-fixation with 4% formaldehyde.

3.3. Histopathology and methods

The brains were fixed *in situ* through saline perfusion through the ascending aorta for 3 min followed by 4% formaldehyde for 10 min and immersion in 4% formaldehyde for 24 h. They were then removed from the skulls by a non-traumatic technique (resection of bone structures at the skull base, followed by a midline incision from the foramen magnum to the nose) and immersion fixed in 4% formaldehyde for more than 24 h. Whole coronal sections of the brains were dehydrated and embedded in paraffin, sectioned at 5 μm with a microtome and stained for RNA/DNA with cresyl violet to visualize damaged neurons. Albumin was demonstrated with the IgG fraction of rabbit anti-rat albumin (Dakopatts, Helsingborg, Sweden) diluted 1:8,000. This reveals albumin as brownish spotty or more diffuse discolorations. Biotinylated swine anti-rabbit IgG was used as a secondary antibody. Then avidin, peroxidase conjugated, was coupled to the biotin and visualized with DAB (diaminobenzidine).

All brains were examined histopathologically by our neuropathologist (A.B.). All microscopic analyses were performed blind to the test situation.

Regarding albumin extravasation, the number of immunopositive extravasates (foci) were recorded under a microscope. None or occasional minor leakage was rated as normal, whereas one larger or several leakages were regarded as pathological. Immunopositive sites were, however, disregarded when localized in the hypothalamus, above the median eminence and laterally including the lateral hypothalamic nuclei, in the immediate vicinity of the third ventricle and just beneath the pial membrane. These structures are well known for their insufficient BBB. Also the presence and distribution of albumin uptake into neurons was judged semi-quantitatively.

Regarding neuronal damage, this were judged semi-quantitatively as no or occasional (score 0), moderate (score 1) or abundant occurrence (score 2) of dark neurons.

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3.4. Statistics

As an initial discriminative test, the occurrence of an effect of exposure (score 1 or higher for albumin foci; score 0.5 or higher for neuronal albumin uptake and dark neurons) was tested against sham exposed controls using Fisher's exact probability test.

The Kruskal–Wallis one-way analysis of variance by ranks was used for a simultaneous statistical test of the score distributions for the five conditions of sham or EMF exposure. When the null hypothesis could be rejected, the non-parametric Mann–Whitney *U*-test for independent samples was used to compare each of the groups of GSM exposed and sham exposed animals.

The occurrence of covariates such as gender, the position of the rat in the TEM-cell (upper/lower compartment) and the TEM-cell used (TEM-cell A, B, C or D) was evaluated using linear regression analysis.

Spearman's non-parametric correlation analysis was used for evaluation of correlation between exposure level, albumin foci, neuronal albumin and dark neurons.

4. Results

In exposed animals there were albumin positive foci around capillaries in the white and grey matter (Fig. 5). The albumin had diffused into the neuropil between the cell bodies, surrounding the neurons, which either contained no albumin or contained albumin in some foci. Scattered neurons were albumin positive. Regarding the dark neurons, cresyl violet staining showed that these were scattered and sometimes grouped within the brain parenchyma.

The occurrence of albumin outside brain vessels was characterized as albumin foci around vessels. After the 7 days recovery time, albumin foci were found significantly more often among exposed rats (25%) than among sham exposed rats (0%) (Fisher's exact probability test, $p = 0.04$). There was a low, but significant correlation between the exposure level (SAR-value) and the occurrence of albumin foci (Spearman analysis, $r_s = 0.33$; $p = 0.04$). Taking the level of exposure and quantification of neuropathological effects into account it could be concluded from a simultaneous non-parametric comparison of all 5 exposure levels with the Kruskal–Wallis test, that the distribution of albumin foci differed significantly (Kruskal–Wallis, $p = 0.012$).

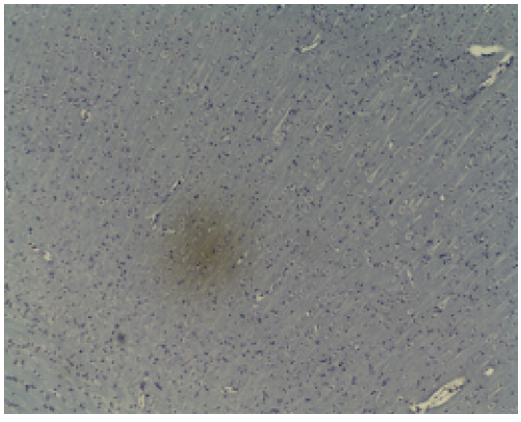

Fig. 5. Focal leakage of albumin shown in brown in the cortex. Albumin: cresyl violet, ×10. GSM-900 EMF exposure at 12 mW/kg. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Pair-wise comparisons between the different exposure levels and sham exposed animals revealed statistically significant differences for SAR of 12 mW/kg (Mann–Whitney, $p = 0.007$), whereas a trend of increased albumin extravasation could be seen for 0.12 mW/kg (Mann–Whitney, $p = 0.1$) and 120 mW/kg (Mann–Whitney, $p = 0.1$).

Also, the occurrence of neuronal albumin was evaluated. A simultaneous analysis for all exposure levels revealed a significant difference between the five groups (Kruskal–Wallis, $p = 0.03$, however Fisher's exact probability, $p =$ ns). A pairwise comparison revealed that albumin uptake occurred more frequently at 1.2 mW/kg as compared to sham exposed (Mann–Whitney, $p = 0.02$). No difference was found for the occurrence of neuronal damage (Kruskal–Wallis, $p =$ ns; Fisher's exact probability test, $p =$ ns).

Linear regression analysis did not reveal any influence of gender, position of the animals in the TEM-cell (upper/lower compartment) or the TEM-cell used (TEM-cell A, B, C or D) on the frequency of albumin foci, neuronal albumin or occurrence of dark neurons.

5. Discussion

The present study provides evidence that GSM exposure results in disruption of the BBB permeability, with remaining, observable effects 7 days after the exposure occasion. Only non-thermal SAR-levels, below the limits of allowed exposure for humans [12] are considered. This finding of increased albumin extravasation after 7 days (Kruskal–Wallis, $p = 0.012$ with all animals included in the analysis, which is also in agreement with the Fisher's exact probability test, $p = 0.04$) is in line with our earlier findings of albumin leakage both immediately following 2 h of GSM exposure [16] and 14 days [19] after 2 h of GSM exposure. Also, the increased occurrence of neuronal albumin 7 days after the exposure is in line with the findings 14 days after exposure [19].

In our previous study, where the animals have been sacrificed immediately after the EMF exposure, we have seen albumin extravasation only at the most in 50% of the identically exposed animals, although all animals are inbred Fischer 344 rats [16]. Among the rats exposed to the pulse modulated EMFs at 915 MHz, 35% showed albumin extravasation. Also in the sham exposed animals, albumin leakage was present (in 17% of the animals). When the animals have survived 7 days after the EMF exposure, albumin extravasation is seen in a lesser proportion (25% of the exposed

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animals) and in none of the sham controls. This could be due to a rapid diffusion of extravasated albumin down to, and beyond concentrations possible to demonstrate immunohistochemically. Numerous routes of clearance of extravasated molecules out from the brain tissue are present in the living brain and compounds can also become sequestered intracellularly, become protein bound or metabolized. After 14 days, albumin extravasation is seen in a somewhat larger proportion of the EMF exposed animals (29% of the exposed animals) and none of the sham controls. Thus, a secondary BBB opening might have started at some time point after the initial opening, leading to a vicious circle of albumin leakage.

The mechanism for the passage of albumin over the BBB is not clear. Extravasation might occur through paracellular routes, including alterations of tight junctions between the vascular endothelial cells, or transcellular routes with induction of pinocytosis or transcytosis, formation of transendothelial channels or disruption of the endothelial cell membrane. In connection to EMF exposure, amplified vesicle-mediated transport across the microvessel endothelium occurs, including also transendothelial channels, but no passage through disrupted inter-endothelial tight junctions [24].

One remarkable observation is that exposure at very low whole-body average power densities gives rise to a pronounced albumin leakage. In the present study, significant effects could be seen already at 12 mW/kg, although the different animal groups included a relatively small number of animals. Most certainly, the trends seen for exposure levels of 0.12 mW/kg and 120 mW/kg would have reached statistical significance if more animals had been included in the different exposure groups.

The phenomenon with increased BBB permeability already at very low energy levels might represent a U-curve response. In our other studies, we have seen that the rats in several of the groups with different SAR-levels of EMF exposure have a significant BBB opening [16,19]. The U-response curve occurs also in connection with other kinds of MW exposure, where cerebral vessel permeability after an initial rise decreased with increasing MW power [39].

Further investigation of BBB permeability in connection to EMF exposure is important not only in order to reduce the potentially harmful effects, but also to use possible beneficial effects [69]. The transport of drugs over the BBB might be regulated, so that targets within the brain can be reached. For example, steering of BBB passage of the antiretroviral agent saquinavir has been accomplished in an *in vitro* model of the human BBB, where a frequency of 915 MHz generated the highest BBB permeability [69]. This could be extremely important in order to reduce the HIV replication in the brain of HIV-infected individuals.

6. In conclusion

The time between EMF exposure and sacrifice of the animals is of great importance for the detection of albumin foci. Seven days after 2 h of GSM mobile phone exposure, there is still an increased permeability of the BBB of exposed rats. This is in concordance with earlier findings of albumin extravasation out into the brain parenchyma immediately and 14 days after 2 h of mobile phone exposure.

7. General conclusion

Taken together, it can be concluded that in a number of studies MW effects upon the BBB permeability have been observed. Increased permeability can be seen both immediately after exposure, but also 7 days after the exposure, as reported in this primary report, and after 14 days. It seems that the effects of the MW radiation might result in persistent changes, such as those seen in our own studies with neuronal damage both 28 and 50 days after 2 h of mobile phone exposure. In a future perspective, with increasing number of active mobile phone users, passive mobile phoning, radiation emitted from base stations and also MWs emitted from other communication sources, effects of low non-thermal levels of exposure must be considered further. The effects seen in the rat studies give some clues about what might possibly happen in the human brain, with a BBB very similar to that of rats. While awaiting latency periods long enough for adequate epidemiological interpretations, further studies on both animals and cells are of utmost importance.

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