Local Exposures to 900-MHz GSM Microwaves Induce Plasma Protein Extravasation in the Rat Brain at Non-thermal SAR Levels

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SUMMARY

Background Non-thermal inflence of GSM radiofrequencies on the blood-brain barrier is controversial. Our aim was to investigate whether non-thermal exposures of the heads of rats to 900-MHz GSM mobile phone microwaves (0.15 to 2 W/kg brain-averaged specific absorption rate (SAR); 10- to 120-minute exposure duration) could lead to plasma protein extravasation in the brain parenchyma.

Methods Before exposure or sham-exposure, animals were progressively trained to contention in order to minimize stress effects. Stress was estimated under exposure conditions by continuous monitoring of arterial blood pressure. The possible effects were also studied by comparing extravasation in rats exposed either awake or under deep anaesthesia. Rats were infused intraveinously with a solution of bovine serum albumin (BSA) combined with fluorescein-isothiocyanate (FITC) at the beginning and at the end of the experiment. Positive controls (intra-carotid infusion of hyperosmolar solution), negative controls (rats not exposed, and not immobilized) were also examined for BSA extravasation. Exposed and sham-exposed animals were killed by an overdose of barbiturics at the end of the exposure. Their brains were perfused and prepared for estimation of BSA extravasation by indirect immunohistochemistry.

Findings 40-minute to 2-hour exposure to GSM microwaves at SAR levels ranging from 0.5 to 2 W/kg gave rise to blood-brain barrier permeabilization to BSA. Extravasation was weak and very localized at the lowest SAR level, marked and broadly present in many brain areas at the highest SAR level. These effects cannot be attributed to other experimental conditions, as shown by the absence of extravasation in sham-exposed animals and by BBB

permeabilization in animals exposed under anaesthesia. They are thus likely to be caused by an action of GSM microwaves exposure on endothelial cells.

Interpretation We postulate that these events, if they occur repeatedly over a long period of time in the human brain, might become a health hazard because of possible accumulation of brain tissue damage and/or hazardous substances in brain tissue.

Acknowledgments:

This work was supported by the CNRS (UMR 5017, UMR 5501), the French Ministry of Research (COMOBIO programme), Bouygues Telecom, and France Telecom R&D. Special thanks to Emmanuelle Haro and Pierre-Emmanuel Dulou for excellent technical assistance and to Bernard Veyret for reading and correcting this manuscript.

Keywords:

Blood-brain-barrier, GSM, microwaves, rat, in vivo, histofluorescence.

INTRODUCTION

The blood-brain barrier (BBB) restricts the entry of blood-borne molecules into the brain, allowing brain tissue homeostasis. This capacity is mainly due to the presence of tight junctions (*zonulae occludens*) between adjacent endothelial cells and to the relative paucity in pinocytotic vesicles of these cells lining cerebral arterioles, capillaries, and venules. Several kinds of stimuli can alter the permeability of the BBB, e.g., acute increase in arterial blood pressure beyond the autoregulatory capacity of cerebral blood flow, circulating hyperosmolar solutions, various inflammatory mediators, activation of blood cells such as leukocytes[1] (Mayhan, 2001). However, the cellular pathways which contribute to the permeabilization of the BBB under these conditions have not yet been entirely elucidated.

Starting in the late 1970's, the interaction of the BBB with microwaves has been intensively investigated. Most of the oldest studies showed an increased permeability of brain blood vessels, some of them at relatively low power levels (0.3 to 5 W/kg) which are unable to induce marked temperature increase in the brain[2,3,4,5,6,7], but some studies have shown no effect[8,9]. It should be noted that the tracers used for estimating BBB permeability in both the Merritt and Preston's studies[8,9] (mannitol and sucrose) were not tested with positive controls and the authors were unable to reveal any increase in BBB permeability at thermal levels of exposure.

More recently, the possible effects of global system for mobile communication (GSM) microwaves on BBB permeability have been studied. Various methods were used either for exposure of the animal (whole-body or head-only, various durations of exposure) or for microwaves (frequencies ranging from 900 to 2450 MHz, pulsed or continuous). The results of these recent studies are contradictory. Most authors report a weak to marked increase in

BBB permeability in the 0.3-5-W/kg range[10,11,12,13,14,15]. Only Tsurita et al.[16] found no effect at an estimated SAR of 2 W/kg in the head (0.25 W/kg whole-body SAR).

The present study is a reappraisal of this problem using well-defined experimental conditions for the estimation of SAR during head exposure as well as for the possible influence of stress during exposure (monitoring of arterial pressure, anaesthetized controls). Effects of microwaves on BBB permeability were tested in a SAR range of 0.15 to 2 W/kg (brain averaged) for exposure durations varying from 10 to 120 minutes.

METHODS

Eighty-one male Sprague-Dawley rats (400-450 g, Charles River, l'Arbresle, France) were used in these experiments. They were divided into ten groups: 1) control (n = 3); 2) sham-exposed (n = 36); 3) exposed at 2 W/kg for 2 h (n = 6); 4) exposed at 1 W/kg for 2 h (n = 6); 5) exposed at 0.5 W/kg for 2 h (n = 6); 6) exposed at 0.15 W/kg for 2 h (n = 6); 7) exposed at 2 W/kg for 40 min (n = 6); 8) exposed at 2 W/kg for 10 min (n = 6); 9) exposed at 2 W/kg for 2 h under anaesthesia (n = 3); 10) positive controls for protein extravasation (n = 3).

After one week of daily progressive habituation to contention in the exposure system (see fig. 1), the left femoral vein and artery were cannulated with polyethylene (PE 50) catheters under ketamine (Sigma K2753, 60 mg/kg) and xylazine (Sigma X1251, 15 mg/kg) anaesthesia. The free ends of the catheters were exteriorized on the back of the neck. One day after catheterization, the awake rats were exposed using a loop antenna placed over their head and shifted by 10° relative to the longitudinal midline in order to expose one cerebral hemisphere more than the other. Briefly, it consists of two parallel lines on a printed circuit board, ending

in a rectangular loop (3 x 1 cm). The antenna is fed in the middle, thus acting as a resonator at 900 MHz. It is tuned to 900 MHz by means of a sliding shorting element, using a network analyzer (HP 8510). The setup is powered by a 10 W 900-MHz, 217 Hz pulsed, GSM generator (RFS 900, RFPA, France), coupled to a 50 dB broadband amplifier (15100B, Eaton). The specific absorption rates (SARs) were determined experimentally using either a Vitek probe for the measurement of temperature rises or an electric field probe. Finite difference time domain (FDTD) calculations were done on homogeneous and non-homogeneous phantoms and the agreement among the three independent methods was very good [17]. For one watt input at the connector, the brain averaged specific absorption rate (BASAR) was 4 W/kg. Local SAR levels were also measured in the brains of 3 freshly-killed animals placed in the exposure system. Just after the death obtained by an over-dose of anaesthetics, a thermistor probe (1mm diameter) was placed in the brain at the vertical of the antenna using a hypodermic needle which was introduced upward through the palate, the base of the skull, and the brain. The needle was removed before measurements, leaving the probe in place. For one watt input at the connector, local SARs ranged between 8 and 8.5 W/kg at the upper location of the thermistor (i.e., in contact with the upper internal side of the skull, immediately under the antenna), 6 to 6.5 W/kg 5 mm below (approximately in the centre of the brain) and 3.8 to 4.2 W/kg at the base of the brain (1 cm under the highest location), this indicating that the BASAR in the real brain could be slightly higher than that estimated in phantoms. However, the BASAR levels specified below are those obtained in models.

In these experiments, the BASAR level in exposed rats ranged from 0.15 to 2 W/kg. Sham-exposed rats were maintained under identical experimental conditions except for exposure to microwaves. The durations of exposures (or sham-exposures) were 10, 40 and 120 minutes. The shortest durations were tested only at a BASAR of 2 W/kg. Arterial blood pressure was continuously monitored in all animals during exposure or sham-exposure. At the

beginning of the exposure (or sham-exposure) and 15 min before its ending, a solution of bovine serum albumin bound to fluorescein-isothiocyanate (BSA-FITC, Sigma A9771, 2 x 12 mg/kg) was infused via the veinous catheter. In order to exclude the possible effects of immobilization stress, 3 rats were exposed to microwaves under urethane anaesthesia (Sigma U2500, 1.5 g/kg i.p.) in the same way as conscious rats. Positive controls of BBB disruption were obtained by hyperosmotic shock (n = 3). Under urethane anesthesia, both internal carotid arteries of these animals were carefully exposed under a binocular microscope. They were then cannulated with Hamilton needles (external diameter 0.3 mm) linked to a microinjection pump (CMA Microdialysis). Then, 1.6-M D-mannitol (Sigma M4125) dissolved in 0.9% saline was infused into both internal carotid arteries at a rate of 1 ml/min for 20 minutes, and BSA-FITC was administered i.v. (Richmon et al., 1998).

At the end of all experimental procedures, rats were killed with an overdose of anaesthetics and the ascending aorta was immediately cannulated for perfusion fixation of the head. They were perfused with 200 ml of cold heparinized phosphate buffer saline (PBS, pH 7.4, 0.1 M), followed by 500 ml of cold 4% paraformaldehyde in PBS solution at a controlled pressure of 140 mmHg. After decapitation, the skulls were opened, the brains removed and post-fixed overnight at 5°C by immersion in the same fixative solution as above and placed for 48 h in a 30% solution of sucrose in PBS at the same temperature. They were then frozen in isopentane (2-methylbutane, Fluka 59065) at –80°C and sliced using a cryomicrotome (coronal sections, 40 µm-thickness). Sections were mounted on polylysin slides and treated for indirect immunohistochemistry against BSA in order to increase the intrinsic fluorescence of BSA-FITC.

All histochemical procedures were performed at room temperature. Brain sections were rinsed in PBS six times for 10 min, incubated in PBS+ (0.2% tritonX100 + 2% normal sheep

serum) for 1 h and then incubated in polyclonal anti-bovine serum albumine antibody (Biogenesis 0330-1104 developed in rabbit, diluted 1/400 in PBS+) for 45 min. After rinsing three times in PBS for 10 minutes, they were incubated in the secondary antibody (Anti-rabbit IgG Biotin conjugate, Sigma B8895, diluted 1/1000 in PBS+) for 1 h and rinsed three times in PBS for 10 min, followed by 30 min incubation in ABC reagent (Vector Laboratories, ABC kit PK-6100). After rinsing in PBS, samples were incubated for 20 min with streptavidin, Alexa Fluor 488 conjugate (Interchim, molecular probes S-11223, diluted 1/1000 in PBS+). They were rinsed extensively with PBS, then dried and covered with Vectashield (Vector, H 1000).

Preparations were examined using a Leitz DMRX fluorescence microscope equiped with a cooled CCD video camera (Photonic Science) and a computer-controlled motorized stage allowing optical tomography (Prior) for acquisition of digitized images. This made it possible to obtain highly contrasted images even of weakly fluorescent elements by successive integration. Images were obtained using a X10 objective (0.445-mm^2 camera field). BSA extravasation was estimated in a simple blind way by direct examination of sections from unknown animals under the microscope.

RESULTS

In the rats placed in the rockets (container), mean arterial blood pressure varied from 95 to 135 mm Hg, (117 ± 2.7 mm Hg, mean ± SE). In none of the rats was the recorded upper limit of arterial pressure compatible with a loss of autoregulation of cerebral blood flow which could lead to an opening of the BBB (see[1] for review). There was no significant difference between mean arterial pressures of sham-exposed and exposed animals (Student's

t-test).

Fluorescence microscope examination showed no noticeable BSA-FITC extravasation in the brain parenchyma of both control and sham-exposed rats (Fig. 2a), while positive controls (hyperosmotic shock) showed a widespread and intense extravasation of BSA-FITC (Fig. 2b).

Two-hour exposures to GSM microwaves at 2 W/kg induced a marked BSA-FITC extravasation in both conscious (Fig. 2c) and anaesthetised rats (Fig. 2d). Fluorescence was particularly visible in the more exposed hemisphere (right) in areas directly located under the antenna. In this hemisphere, the deep grey matter such as hippocampus and central grey nuclei (putamen, caudate) as well as the frontal cortex were heavily labelled, while the upper layers of the parietal cortex showed no or few fluorescent elements. However, all brain structures had some labelling, including the cerebellum. Most of the BSA-FITC diffusion within the brain parenchyma was restricted to the immediate vicinity of brain microvessels (see Fig. 3).

After 2 h exposures of conscious rats at 1 W/kg (Fig. 2e) and 0.5 W/kg (Fig. 2f) the presence of BSA-FITC was still detected in the brain parenchyma, but it was mainly restricted to the deep gray matter located under the antenna and to the frontal cortex, apparently in lesser amounts than after exposure at 2 W/kg.

At the lowest SAR level tested in these experimental series (0.15 W/kg), a 2 h exposure did not induce any visible BSA-FITC extravasation in the brain parenchyma.

A 40 min exposure at 2 W/kg resulted in an extravasation of BSA-FITC comparable to that obtained after a 2 h exposure at the same SAR (Fig 4). However, 10 min exposures at 2 W/kg did not lead to any visible extravasation.

DISCUSSION

The present study shows that at the high and moderate BASAR levels of 2, 1 and 0.5 W/kg, respectively marked to weak protein extravasation in the brain parenchyma are observed. At these levels, an elevation of temperature in any part of the brain is most unlikely. Measurements in homogeneous phantoms indicated that the highest increase in temperature to be expected in alive animals could not exceed 0.1° C at the level of dura mater, just below the antenna and our direct measurements in the brain of 3 freshly killed animals (i.e. in the absence of thermal clearance by blood flow) indicate that this increase might actually be much lower than 0.1° C.

The leakage of protein from brain blood vessels attests alterations of the BBB characteristics which cannot be due to a rise in arterial pressure induced by the contention stress, as shown by arterial blood pressure monitoring and by the fact that at the highest levels of exposure, there was no visible labelling difference in the brain of animals exposed either under anaesthesia or conscious. Furthermore, no noticeable protein extravasation was observed in sham-exposed rats which were submitted to identical experimental conditions. Therefore, even if one cannot totally exclude a weak potentiation of RF effects on the BBB permeation by contention stress, the present results clearly show that BBB characteristics are altered by exposure to GSM microwaves and that, in the limits arbitrarily chosen for this study, extravasation increases correlatively with SAR level and duration of exposure. The fact that there was no evidence of protein extravasation in the brain parenchyma following a 10 min exposure at the maximum BASAR level tested does not ascertain that this duration is

too short for initiating BBB permeabilization. Since all animals were sacrificed just after exposure, whatever its duration, it remains possible that either the permeabilization process or the protein migration are too slow for allowing a significant amount of albumin to reach the extra-vascular compartment within this time lapse. Further experiments will be designed to study whether albumin can be detected in animals allowed to survive for 40 min following a 10 min exposure.

These results are in agreement with some previous studies showing that GSM continuous or pulsed microwaves increase by some extent BBB permeability in living rats or mice[10,11,12,13,14,15]. It should be noticed that both the Fritze and Finnie groups[13,14,15] concluded to a non-significant or negligible effect after a head-mainly or whole-body exposure (of rats and mice, respectively) at non-thermal SAR levels (0.3 –4 W/kg in the head). However, Fritze et al.[13] observed that "in the sham-exposed control group (n = 20), 3 animals exhibited a total of 4 extravasations. In animals irradiated for 4 h at SAR of 0.3, 1.5 and 7.5 W/kg (n = 20 in each group), 5 out of the 10 animals of each group killed at the end of the exposure showed 7, 6 and 14 extravasations, respectively (…)". The proportion of animals of all exposed groups which showed extravasation was thus about twice that of the sham-group and the number of spots of extravasations also increased slightly at the non-thermal levels of 0.3 and 1.5 W/kg. In the same way, Finnie et al.[15] reported 26% of extravasation in controls, 65% at 0.25 W/kg (whole-body), 51 % at 1 W/kg, 78 % at 2 W/kg and 20% at 4 W/kg. Only the exposure at the borderline of temperature elevation (4 W/kg) seemed to have no influence. One may wonder why, in these two sets of experiments, so many extravasations were observed in sham-exposed groups. This could be due to contention stress since no physiological parameter allowing to evaluate the stress level was measured during sham-exposure or exposure. Whatever the reason was, this abnormally high level of "spontaneous" extrasavation reduced

dramatically the probability of obtaining statistical significance in relatively small samples. In our experimental protocol, the one-week progressive training to contention seemed to prevent this problem, since monitoring of arterial blood pressure did not show any abnormal rise and since no extravasation was seen in our sham-exposed animals.

Finally, the study of Tsurita et al.[16] was the only recent one concluding without any ambiguity to an absence of effects of microwaves on the BBB at non-thermal levels after exposure of rats to a 1400 MHz signal for 1 h per day during 2 or 4 weeks at 0.25 W/kg (whole-body, i.e. 2 W/kg BASAR).

Moreover, a recent in-vitro study[18] indicated that GSM microwaves at 1.8 GHz (0.3 W/kg) modified the metabolism of endothelial cells in co-culture of rat astrocytes and porcine brain capillary cells in a way which significantly increased the permeability of these cells to ^{14}C-sucrose compared to sham-exposed cells. The mechanism responsible for the increased permeabilization could be a rise in pinocytotic transportation of tracer through these cells. Under normal conditions, pinocytosis is particularly weak in cerebral endothelial cells.

A similar mechanism had already been proposed by Neubauer et al.[10] who showed *in vivo* that exposure-induced extravasation was decreased by previous administration of colchicine. It is well known that microtubules and actin are required for efficient transcytosis and that colchicine can reduce transcellular transportation of proteins by inducing the disassembly of microtubules.

Whatever the precise endothelial cell mechanism involved in the cerebrovascular permeabilization to proteins and other molecules, it seems that its occurrence could be due to a direct influence of GSM microwaves on cerebral endothelial cells and/or surrounding glial cells and neurons. In a recent in-vitro study, Leszczynski et al.[19] showed that one-hour exposures to GSM microwaves at 2 W/kg (with no temperature increase) induced an

increased expression and phosphorylation of heat shock protein 27 (HSP 27) in cultures of human endothelial cell lines. Other changes in expression/phosphorylation were observed by these authors in numerous although not yet identified proteins after microwave exposure. Preliminary results obtained *in vivo* in our laboratory also suggest that GSM microwaves (1 W/kg BASAR, 40 min exposure) can induce changes in protein synthesis by brain cells (decreased or increased synthesis). Particularly, we observed these changes in several HSPs among which HSP 27, whose expression was significantly increased. Such changes in HSPs expression could play a prominent role in the BBB permeabilization by GSM microwaves through stabilization of endothelial cell stress fibers, as stated by Leszczynski et al.[19] based on the work of Landry and Huot[20].

Even if transient, a permeabilization of the BBB caused by exposure to GSM microwaves may have potentially important consequences for the health of GSM mobile phone users. Beside the entrance in the brain parenchyma of undesirable molecules such as proteins, ions, metals, or some neurotransmitters agonists normally or accidentally present in the blood plasma, which can lead to various brain dysfunctions or pathologies, it cannot be excluded that it also allows virus penetration.

Even if these molecules have no direct influence on brain functioning, protein extravasation inevitably leads to brain oedema, due to change in osmotic pressure between intra- and extra-vascular compartments. Repeated oedemas can induce glial and neuronal damage in the vicinity of microvessels.

It remains to determine whether this extravasation also occurs in human being, but numerous indirect evidence such as headache complaints or changes in EEG patterns after phone use indicate that this could be the case. Actually, migraine has repeatedly be said to originate in permeabilization of meningeal blood vessels and/or in BBB alterations and it can

be speculated that several blood-borne molecules can modify neuron electrical activity when penetrating into the brain parenchyma. If this is the case, our results may indicate that pathological events would occur at moderate or high SAR levels and after relatively long phone communications.

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LEGENDS TO FIGURES.

Figure 1 :

Schematic drawing representing a rat in the contention system with the antenna in place.

Figure 2 :

Representative pictures of BSA extravasation in different experimental conditions.

All images represent approximately the same area (camera field; x10 objective) of similar brain coronal sections (thickness: 40µm, inter-oral 9.5-10 mm[21]). This camera field includes mainly areas 1 and 3 of the frontal cortex of the right hemisphere, directly under the antenna.

*: upper surface of the brain.

a: sham-exposed animal; b: positive control (infused with hyperosmolar solution); c: exposed 2 h at 2 W/kg; d: exposed 2 h at 2 W/kg under anaesthesia; e: exposed 2 h at 1 W/kg; f: exposed 2 h at 0.5 W/kg.

White dots represent extravased BSA revealed by indirect immunofluorecence.

Filled arrows : arterioles or veinules. Opened arrows : microvessels.

Figure 3 :

Detail of BSA extravasation around microvessels.

Two hours exposure at 2 W/kg.

At a higher magnification (objective x 40), BSA appears to be localized mainly in the immediate vicinity of microvessels (filled arrows) and spreading in the brain tissue seems to be relatively limited (opened arrow).

*: empty lumen of microvessels, probably veinules and/or capillary.

Figure 4 :

Representative picture of BSA extravasation after 40 min exposure at 2 W/kg.

a: sham-exposed animal; b: exposed animal.

Notice that at this SAR level, BSA the amount of BSA is important after only 40 min of exposure. Double blind comparison between brain sections from rats exposed 2 h and rats exposed 40 min, respectively, did not allowed us to differentiate two levels of extravasation by visual estimation.

* : upper surface of the brain.

Contributors

Fatma Töre was the researcher in charge with animal experiments and immunohistochemistry techniques. She participated in the analysis of histological preparations and contributed in writing the report. **Jean-Louis Lavie** designed and realized the system allowing continuous arterial blood pressure measurements in restrained conscious rats. **Pierre Aubineau** designed the experimental protocol, contributed in the set up of the first experimental phase and in the blind analysis of the histological preparations. He also wrote the report.

Conflict of interest statement

Pierre Aubineau received research grants from Bouygues Telecom and France Telecom R&D. **Fatma Töre** was employed one year (over the three years of this study) on fundings provided by Bouygues Telecom and France Telecom.

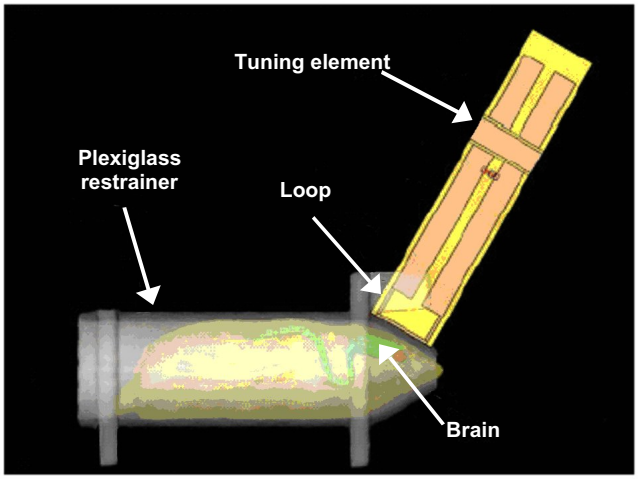

Figure 1

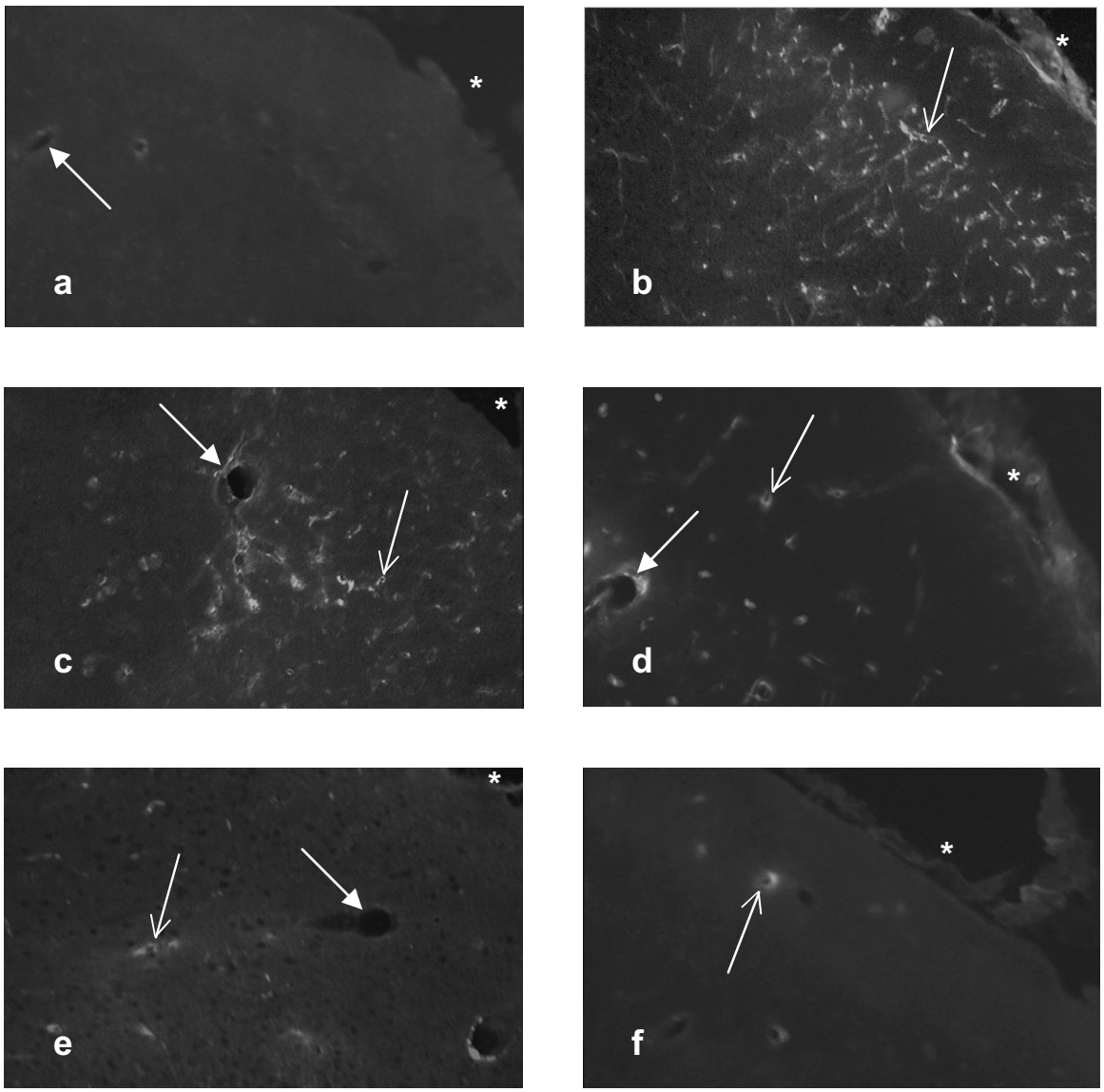

Figure 2

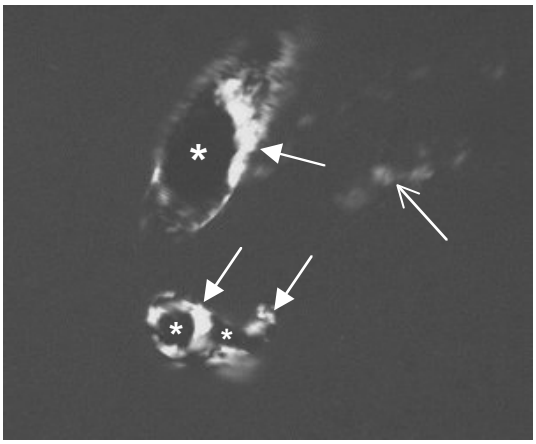

Figure 3

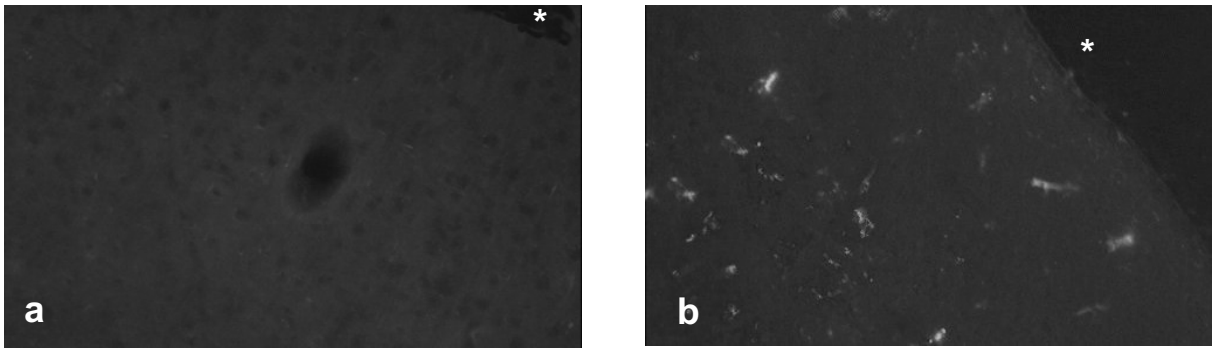

Figure 4