Blood-brain barrier disturbances and morphological changes in rat brain after photochemically induced focal ischaemia

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INTRODUCTION

Acute disturbances in cerebral circulation comprise an often encountered and clinically important problem. Focal cerebral ischaemia resulting in brain infarct is a well-defined entity among other diseases in this group. Infarctions are formed as a result of acute ischaemic insults and comprise severe, often irreversible damage of neurones and focal tissue necrosis surrounded by reactive gliosis. Several experimental methods have been devised to study the pathological processes and pharmacotherapy of ischaemic infarcts in the brain.

One of the first experimental models comprised mechanical blockade of large arteries supplying the brain. This was most commonly achieved by a ligation of the medial cerebral artery [1, 4, 7, 12, 17]. However, the main drawback of this approach is the considerable degree of invasiveness of this complicated neurosurgical procedure. Moreover, the degree of ischaemia and tissue necrosis varies due to interindividual anatomical variability in arterial blood supply and collateral circulation. As shown by us in Mongolian gerbils (unpublished)...
and in other species, there is a considerable structural variation in the network of anastomoses connecting the cerebral medial and posterior arteries.

Another approach was to induce brain ischaemia by intraarterial injections of microspheres or autologous blood clots [8, 9]. Although the advantage of these models is a closer relationship to the pathogenesis of brain infarcts in humans, the degree of ischaemia cannot readily be standardised, mainly due to interindividual variability in the anatomy of cerebral blood vessels. Taking a rabbit model as an example [10], there are considerable variations in the scheme of arborisation of the commune carotid artery into the external and internal carotid arteries.

A new generation of experimental models comprises photochemical techniques, where the surface of the brain is irradiated following the intravenous administration of rose bengal. The photochemical reaction induced by photon energy in the exciting radiation releases free radical species (mainly singlet oxygen) responsible for peroxidation of membrane lipids and proteins. In the next phase, platelet aggregates are formed which obliterate brain capillaries and trigger focal ischaemic infarct of a reproducible size and depth. This model is potentially useful for the evaluation of pharmacological interventions aiming at the amelioration of the effects of brain ischaemia.

The aim of this work was to describe and evaluate the usefulness of the newly developed model of photochemically induced brain ischaemia and to compare it with other methods reported in the literature.

**MATERIAL AND METHODS**

Sixteen male Wistar rats, weight 150–200 g, age 2–2.5 months, were anaesthetised by intraperitoneal injection of 325 mg/kg 3% solution of chloral hydrate. Rose bengal (3% solution in physiological saline) was injected into the zygomatic vein at 40 mg/kg. The skin of the parietal region was incised in the midline after the periosteum was removed and the exposed parietal bone was irradiated via the fibre optic. The irradiated site was the region over the left hemisphere in the middle distance between the coronary and lambdoid sutures, slightly lateral to the sagittal suture.

The light source constituted an air-cooled 250 W halogen bulb and the photon energy was transmitted by a fibre optic covered with an elastic metal coating. The irradiating apparatus was custom-made in the Institute of the Brain, Russian Academy of Medical Sciences, in the laboratory of J.W. Wiktorow. The construction of the equipment allowed for a stable placing of the fibre optic outlet over the predetermined area of the skull. The irradiated area was cooled by water dripping slowly from a thin tube fixed to the fibre optic.

After a 30-min irradiation period, the animals were kept in standard housing conditions. The material for morphological studies was sampled in groups of 3 rats at 24 h, 4 days and 1 week after irradiation. The animals were anaesthetised with chloral hydrate as described above and perfused by an intracardiac injection of a mixture of 4% formaldehyde, glacial acetic acid and 96% ethanol (2:1:7 v/v/v). The brains were removed 2 h after perfusion and dehydrated in the increasing alcohol gradient (96% overnight, absolute anhydrous alcohol 2 × 30 min). Tissue blocks of 5 mm thickness were isolated by cutting in the frontal plane adjacent to the anterior and posterior sites of macroscopically discernible area of necrosis. The samples were incubated in a mixture of absolute ethanol and chloroform (1:1, then 1:2 v/v) and in pure chloroform and finally embedded in paraffin.

Ten µm microtome sections were stained with haematoxilin-eosin (H&E), acid vanadium fuchsin (AVF) and cresyl violet (CV). To determine the degree of gliosis, the paraffin sections were stained for GFAP in astrocyte processes according to the method of Hsu et al. [6].

The integrity of the blood-brain barrier was assessed by determining the area of the extravasation of a barrier marked Evans blue to brain parenchyma. The animals that survived 24 h after irradiation were injected intravenously with 1 ml of filtered 2% Evans blue solution in physiological saline. Animals were decapitated immediately after 2 h after injection. The isolated brains were fixed in absolute ethanol for 1 day and photographed to visualise the marker on the surface of the cortex. Then the brains were cut in the frontal plane through the field of necrosis in order to determine the depth of the stained area. An immunohistochemical staining procedure was employed to determine the barrier function at the microscopic level. Extravasated endogenous albumin was stained in 10 µm paraffin sections by a specific antibody (Dako A/S, Glostrup, Denmark) at a dilution of 1:200. The sections were additionally stained with haematoxilin and embedded in Canada balsam (Serva).

The control material was obtained from 3 rats that were not injected with rose bengal but which were irradiated like the animals in the experimental groups. The transcardiac perfusion was performed 4 days after irradiation. Other procedures were identical to those used in the experimental groups.

During irradiation all animals were placed on a table thermostat at 37°C.
RESULTS

Control group

The brains of 3 control rats irradiated transcranially without the preceding rose bengal administration did not show any features of brain damage, neither on the brain surface nor on the frontal sections. At a magnifying glass magnification no features of hyperaemia or tissue damage were observed in the vessels of pia mater under the irradiated field. In stained histologic sections both cerebral cortex and subcortical structures remained normal.

The GFAP staining was weak and revealed single, weakly arborised astrocytes at the lining of cerebral ventricles, in the molecular layer of the cerebral cortex and in the fibrillar elements in the white matter (Fig. 1). More pronounced aggregates of moderately stained astrocytes were seen in the stratum lacunosum—molecular of the hippocampus (Fig. 2).

Experimental group

In all animals features of brain damage were observed and the degree of structural changes depended on the time of survival after irradiation. At the macroscopic level the areas of pale tissue, slightly larger than the initial irradiation field, were readily discernible against the background of normal surrounding tissue.

In the H&E and CV-stained material sampled from the animals that survived 24 h after irradiation, we observed single reactive cells and weak or negative staining of the neurones. Parenchymal oedema was present in the tissue surrounding the necrotic focus, manifested as loosening of the fibre structure in the corpus callosum (Fig. 3).

No morphological alterations were observed in the subcortical structures, the cerebral cortex distant to the region of necrosis, or in the contralateral (right) hemisphere.

Four days after ischaemia the field of necrosis was surrounded by a well-formed ring comprising reactive cells. The microglia cells were most abundant close to the necrosis, whereas reactive astrocytes were most prominent at the periphery (Fig. 4, 5). A moderate level of oedema was still present, though less pronounced than in the animals 24 h after the irradiation. Shrunk, hyperchromatic cortical neurones were discernible near the necrotic region (Fig. 6), in the CA1 sector (Fig. 7) and in the hippocampal dentate gyrus (Fig. 8). In regions distant to the irradiated area, normal structure of the cerebral cortex was seen (Fig. 9).

The immunocytochemic GFAP reaction in the 24 h group was similar to that seen in the control animals. There were slight differences in the hippocampus and in the molecular layer of cerebral cortex that comprised an increased number of stained astroglial processes and the presence of a positive, granular staining within the region of necrosis. Significant differences were noted in the animals that survived 4 and
Figure 4. A well-developed reactive cells ring surrounding the field of necrosis. 4 days after irradiation, H&E, × 40. ▶ area of necrosis, ● the reactive cells ring separating necrosis from the surrounding tissue.

Figure 5. Fine structure of the reactive cells from Fig. 4. Note a layer of cells surrounded by reactive astroglia. 4 days after irradiation, H&E, × 200. ▶ area of necrosis, * the reactive cells ring separating necrosis from the surrounding tissue, ▶ layer of reactive astrocytes.

Figure 6. Large numbers of dark, intensively labelled neurones, distributed in the middle cortical layers. 4 days after irradiation. AVF staining, × 200.

Figure 7. Interneurones in the CA1 hippocampal sector in the irradiated brain hemisphere stained 4 days after irradiation with AVF, × 200.

Figure 8. Dark neurones distributed along the proximal margin of dentate gyrus. 4 days after irradiation, × 200.

Figure 9. Normal structure of cerebral cortex in a region distant to the irradiated brain area. 4 days after irradiation. CV staining, × 200.
7 days after irradiation. In these cases the proliferation of astrocytes was present not only in the penumbra (Fig. 10, 11) but also in the peripheral regions of the brain cortex and in the contralateral (right) hemisphere.

Assessment of the blood-brain barrier with Evans blue was done on animals at 24 h after irradiation. In the material sampled 2 h after intravenous injection of this dye a macroscopically discernible blue area of extravasation was present on brain surface corresponding to the irradiated region. A similar phenomenon was present on the frontal sections of the brain and the region of extravasation comprised the cerebral cortex and the corpus callosum, often extending to the contralateral site. The region of Evans blue extravasation was larger than that detected with the microscopic staining for autologous albumin. When the material was sampled immediately after injection of Evans blue, a vascular network of pia mater was visualised over the unstained irradiated cortex (Fig. 12).

The immunostaining for endogenous albumin was negative in control animals except for the barrier-deficient brain regions and a small number of capillaries in the cerebral cortex. One day after irradiation the overwhelming proportion of neurones demonstrated positive immunoreactivity with unstained nucleus and intensively stained cytoplasm and axons (Fig. 13). The number of labelled cells was inversely proportional to the distance from the centre of necrosis. At the periphery of the irradiated hemisphere and in the contralateral hemisphere the albumin immunostaining was negative. Similarly to the findings with Evans blue, the albumin immunoreactivity was positive in the corpus callosum and in the deep regions of the unirradiated hemisphere. Four days after the induction of photochemical necrosis the immunoreactivity of endogenous albumin was similar to that seen after 24 h. A recovery in barrier integrity was observed after 7 days, at which point the positive immunostaining was only present in the area immediately adjacent to the necrotic focus and in the walls of a larger number of blood vessels than in control animals.

**DISCUSSION**

The results presented in this study confirm the usefulness of the described method of photochemical brain damage as a model of focal cerebral ischaemia. The crucial parameters: the intensity of light source, the time of irradiation and the dose of rose bengal, seemed to be optimised in view of an insignificant mortality of experimental animals and the reproducibility of the photochemi-
cal reaction. Experiments with Evans blue further confirmed this conclusion. In the material sampled immediately after injection, a complete lack of cortical staining indicated a total occlusion of vessels by platelet aggregates. Disturbances in the haemodynamics in the meningeal vessels constitute the primary cause of the impairment of blood flow in the radial arterioles supplying the cerebral cortex. This pathomechanism of acute brain ischaemia explains the reproducibility of several parameters, such as the localisation and size of the ischaemic region, the size of the necrotic field and the morphological changes in the tissue around the field of necrosis. However, the most important advantage of the photochemical model is that brain ischaemia is caused by capillary obstruction by platelet thrombi, which, along with the minimal invasiveness of the experimental procedure, brings this model closer to the clinical situation.

The observed morphological changes and functional alterations in blood-brain barrier provide information about the dynamics of the development of ischaemic brain necrosis and the abnormalities in the tissue surrounding the focus of ischaemia. The interpretation of morphological changes has been facilitated by the employed method of tissue fixation and staining methods, especially with acid vanadium fuchsin. In our opinion this method is more accurate than the commonly used H&E staining since it more precisely visualises altered neurones, especially in the early phase of cell damage. The quality of stain might be employed to classify neuronal damage into reversible and irreversible ones.

We have not employed horseradish peroxidase technique, which has commonly been used before for assessment of blood-brain barrier damage. Instead, we used the cytochemical method, revealing the presence of extravasated endogenous albumin. According to the suggestion of Vorbrodt & Dobrogowska [13], peroxidase, that is a plant protein phylogenetically distant to mammalian proteins, may initiate pathological reactions in the endothelium, leading to an increase in the permeability of blood vessels [2, 11]. Like any other alien protein, peroxidase is likely to be eliminated actively from brain parenchyma and therefore an extensive standardisation of the dose, the time of administration and the time of tissue sampling would be required. Even small procedural errors may result in large inconsistencies in the results.

We consider the determination of extravasation of endogenous albumin to be the method of choice for the assessment of blood-brain barrier integrity, also when used for electron microscopy [13–15].

An intensive cytochemical reaction in neuronal cytoplasm, seen in the region immediately adjacent to the focus of necrosis after 24 h, declined with time (4 and 7 days after irradiation). This development matched the regions where there were clear-cut haemodynamic disturbances [3]. The data of Dietrich et al. [3] indicate that local perfusion of different brain structures declines in the irradiated hemisphere, most notably in the cerebral cortex. The immediate causes of haemodynamic disturbances are platelet aggregates forming directly in the cerebral cortex of the irradiated brain area. The platelets are able to release different mediators, such as serotonin and thromboxane A₂ [5, 16], which induce vasoconstriction and lead to the augmentation of thrombus formation in the adjacent but unirradiated areas.

The topography and time sequences of the development of haemodynamic alterations in the irradiated and unirradiated contralateral hemisphere [3] are similar to the sequence of the development of morphological changes in the brain and the disruption of the blood-brain barrier. These observations allowed us to speculate that the common reason for all observed morphological and functional changes is abnormal blood perfusion, which, according to Dietrich et al. [3], develops as early as 30 min after irradiation and peaks after 4 hours. The immediate consequences of these processes are increased vessel permeability to proteins, resulting in brain oedema, which is most pronounced 24 h after irradiation.

The morphological changes in cortical neurones and gliosis are the results of tissue acidification that develops in the early phases of ischaemia. Subsequently, the disruption of barrier integrity and brain oedema develop and trigger the development of late pathological alterations in the brain, characteristic for the employed model of photochemical, focal brain ischaemia.
REFERENCES


