Time-dependent axonal impairment in experimental model of brain oedema

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Abstract

OBJECTIVES: Clinically very serious condition of ischaemia and brain injury which are often associated with brain oedema is frequently accompanied by the impairment of the structural integrity of axons. We wondered whether the brain oedema (without ischemia brain injury) can induce structural axonal impairment.

METHODS: Brain oedema was induced by osmotic blood-brain barrier opening with 20% mannitol applied selectively into the internal carotid. Axonal changes were recognized as signs of myelin disintegration (oedematous vesicles, varicosity, myelin fragmentation) at histological sections stained with Black Gold in hippocampal areas CA1 and CA3 and in the dentate gyrus and cerebral cortex at time intervals of one hour, one day, three days and one week after the oedema induction.

RESULTS: Impairment of the structural integrity was identified in myelin sheets in all areas studied in all experimental groups. Whereas in the control group axon were of the uniform diameter, in the experimental groups various forms of myelin disintegration were observed.
The progression of myelin damage depended on the time elapsed after the oedema induction.

CONCLUSION: Opening the blood-brain barrier with an osmotic insult induces brain oedema which represents a factor triggering axonal impairment accompanied with myelin changes. The development of axonal changes initiated by brain oedema only (without ischemia brain injury) is a novel observation.

Abbreviations:
CNS - Central Nervous System
BBB - Blood-brain Barrier
ACC - Arteria Carotis Communis
ACI - Arteria Carotis Interna
ACE - Arteria Carotis Externa
DG - Dentate Gyrus
INTRODUCTION

State of the internal environment of the nervous tissue is the result of instantaneous balance between diverse aspects of neuronal activity, activity of the glial elements, volume and properties of extracellular space and regulated permeability of the blood-brain barrier (BBB) (Pokorný et al. 2002). Structural integrity of the axons is one of the essential conditions of the normal nervous tissue function. Neuronal activity can be severed in clinical pathologies, such as ischemia or injury. Whether such a pathological state is reversible (i.e. can be treated) depends (beside other) on the grade of structural damage done to the axons. The options for experimental study of axonal integrity changes include histological examination of defined regions of the brain after an intervention in the internal environment of the nervous tissue (e.g., a case of brain oedema). In the work submitted here we induced oedema with the method of osmotic BBB opening with 20% mannitol applied selectively into the internal carotid with axonal changes detected histologically after staining with Black Gold. The histological tests were carried out at various time intervals (one hour, one day, three days and one week) after the oedema had been induced by mannitol. The idea was to ascertain the extent of axonal changes relative to the time elapsed from the point when the brain internal environment was disrupted by the induced oedema. Results from experimental animals were compared with those in healthy controls, i.e. brains free from induced oedema (control group).

MATERIAL AND METHODS

For the experiment, we used adult animals of both genders from the Wistar strain of laboratory rats (weight 350–450 g), all treated in accordance with the current Guidelines for the treatment of laboratory animals (EU Guidelines 86/609/EEC).

Microsurgical exposure of the internal carotid (ACI)

Animals were put into the state of general anaesthesia using intraperitoneal application of thiopental in the dose of 4 mg/100 g and allowed to ventilate spontaneously throughout the procedure. Starting from a skin incision along the midline between the upper end of the sternum and the mandible, the whole common carotid artery (ACC, arteria carotis communis) was exposed with a standard microsurgical technique and, beyond its bifurcation, also the proximal portions of the internal carotid (ACI, arteria carotis interna) and external carotid (ACE, arteria carotis externa), which was ligated close beyond the bifurcation. An intraluminal catheter was introduced into the ACC trunk from the arteriotomy for selective application of mannitol. With the application over and the catheter removed, the ACC was ligated distal to and proximal to the arteriotomy. The operation concluded with a single-layer suture (Kozler 2002).

Osmotic opening of the BBB – induction of oedema

Mannitol 20% (200 g in 1 000 ml of water for injection, 1 098 mosmol/l) in a dose of 5 ml/kg was selectively

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Fig. 1. Diagram of AP-plane frontal section of the brain, 2.5 mm posterior to the bregma. Regions of the myelin changes evaluation are labeled with numbers.

- hippocampal area CA1 (CA1)
- hippocampal area CA3 (CA3)
- dorsal blade of the dentate gyrus (GD)
- parietal cerebral cortex (PC)
- temporal cerebral cortex (TC)
Axonal impairment in brain oedema

applied in the ACI at a rate of 0.12 ml/sec (Saris et al. 1988, Rapoport 2000). After the surgical intervention, the animals were placed in boxes offering standard access to food and drink.

**Perfusion and fixation**

Animals were sacrificed in a deep anaesthesia via standard transcardial perfusion with a 4% solution of paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 minutes. After removal from the skull, the brain was fixed in the same solution for 24 hours. Serial coronary sections (30-µm thick) were made in a vibrotome from each brain, placed on gelatin-coated slides and dried.

**Neurohistology**

The sections were rehydrated and axonal changes detected with the Black Gold II method of staining (Histo-Chem Inc., Jefferson, AZ, USA.) (Schmued et al. 1999). The hippocampal formation was the main part of the brain under study because of its known high sensitivity to various pathogenic stimuli. Analysis was centred on the CA1 and CA3 areas of the hippocampus and on the dorsal blade of the dentate gyrus (DG). The results were compared with the adjacent parts of the parietal and temporal cortex (Figure 1).

The neurohistological picture of the structural integrity of the axons was assessed with the aid of the following grades of myelin degradation: 1 = no change, 2 = sporadic oedematous vesicles and sporadic oedematous axons, 3 = multiple vesicles, varicosity, oedematous axons and helical course of axons, 4 = myelin fragmentation.

Neurohistological examination was done in four experimental and one control group, each consisting from three rats. For the statistical analysis data from 10 sections of each brain were used. Experimental groups: brains were perfused and fixed at one hour (group 1H), at 24 hours (group 1D), at 72 hours (group 3D) and at a week (group 1W) after the oedema induction by

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**Fig. 2.** Myelinated axons in stratum granulosum and adjacent stratum moleculare and stratum plexiforme of gyrus dentatus.

- Empty arrow – oedematous vesicle; Full arrow – oedematous axon; Empty arrowhead – fragmented myelin; (original magnification 500 times, scale bar represents 50 µm)

- a) Control animal – axons are of the uniform diameter without varicose distensions
- b) Myelin changes evaluated as grade 2 (sporadic oedematous vesicles and sporadic oedematous axons).
- c) Myelin changes evaluated as grade 3 (multiple vesicles, varicosity, multiple oedematous axons)
- d) Myelin changes evaluated as grade 4 (multiple vesicles, varicosity, myelin fragmentation)
mannitol. Control group (CG): brains were perfused without the oedema induction. Brain areas with lower density of myelinated fibres (e.g. stratum granulosum, stratum pyramidale) were preferably used for evaluation and documentation.

**Statistical analysis**

The results were statistically evaluated using the t test and one-way analysis of variance (ANOVA) followed by Dunnett post hoc analysis. The statistical software GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses.

**RESULTS**

With the general anesthesia over, all animals in the experimental groups (except group 1H, perfused during the anesthesia) showed standard behaviour without any discernible neurological deficit.

Impairment of the structural integrity was identified in myelin sheets in all areas studied in all experimental groups. Whereas in the control group axon were of the uniform diameter without varicose distensions (Figure 2a), in the experimental groups we observed myelin changes classified as grades 2 to 4 (Figure 2b,c, d).

Data from the quantitative assessment of the histopathological changes of myelinated axons were summarized for each experimental group. The data collection and evaluation is demonstrated at Figure 3. In this example, animals were perfused one hour after the oedema induction (group 1H) and axonal changes of different intensity were classified according the given scale in CA1 and CA3 hippocampal areas, in the dorsal blade of the dentate gyrus and in adjacent temporal and parietal cortex of the right and left hemispheres. Axonal impairment was more severe in the hippocampal area compared to the cortex. The severity of axonal changes (grades of myelin degradation) varied in this group between 2.0 and 2.75. Data from all experimental and control groups were summarized in Figure 4. The average grade of axonal changes was 1.0 in the control group, 2.5 in group 1H, 2.75 in group 1D, 3.25 in group 3D, and 3.5 in group 1W. The differences between the control and each of the experimental groups were statistically significant ($p<0.0001$).

**DISCUSSION**

Impairment of the structural integrity of axons brings about reversible or irreversible loss of nervous tissue functions. Clinically very serious condition are ischaemia and brain injury which are often associated with brain oedema. Some authors refer to brain oedema as an axonal change-initiating factor (Genneralli 1998; Dirnagl 1999; Sahuquillo 2001). We wondered whether it would be possible to study the course of development of axonal changes under experimental conditions. To induce oedema we used the method of osmotic BBB opening with mannitol selectively applied into the internal carotid. Opening the BBB with an intra-arterially applied hypotonic solution was initially described as an undesirable side effect of contrast medium application during cerebral angiography (Broman & Olsson 1949). The idea of opening the BBB by an osmotic insult was first accomplished by Rapoport (Rapoport 1970). The trick of opening the barrier in response to one-off intracarotid administration of a hyperosmolar solution rests in the shrinkage of endothelial cells causing tight junctions to expand up to an average of 20 times their normal diameter (Rapoport 2000). This mechanism of BBB opening results from vasodilatation in the cerebrovascular bed, dehydration of endothelial cells and contraction of their cytoskeleton under the impact of the osmotic insult alone, without the participation of any other energy-dependent mechanisms of BBB permeability increase, e.g., vesicular transport (Greenwood et al. 1988; Rapoport 2000).
In the experimental group perfused one hour after the induction of oedema (1H), the effect of general anaesthesia still persisted. It was therefore not possible to assess the animal behaviour in this particular group. In experimental groups 1D, 3D and 1W with the effect of general anaesthesia completely abated, all animals exhibited standard behaviour, eating and drinking as usual, free from any discernible neurological deficit. This kind of behaviour appears to reflect the use of osmotic BBB opening, a rapid, short-term and reversible effect. The osmotic insult opens the BBB within 30 seconds and keeps it open for the period of 10 minutes. Thereafter, increased BBB permeability can no longer be proved (Lin et al. 1997; Lafuente et al. 1990; Rapoport & Robinson 1986, Robinson & Rapoport 1987). The efficacy of this method had been verified using markers (Evans Blue and sodium fluorescein) already in our previous experiments (Kozler & Pokorný 2003a,b). In our view, axonal changes in the experimental groups (1H,1D, 3D and 1W, (grades 2 and 3 and 4 of our rating scale) were sufficiently manifested to be regarded as the evidence of initiated disruption of axonal structure integrity caused by the loss of homeostasis of the internal environment of the nervous tissue under the impact of induced oedema. The progression of axonal changes in the experimental groups (1H, grade 2.5; 1D, grade 2.75; 3D, grade 3.25; and 1W, grade 3.5) points to the progressive development of the cellular component (p<0.0001). Our method of osmotic insult opens the BBB reversibly (see above) only for a brief period (few minutes). However, damage of the axonal structure progressed even after the period of increased BBB permeability was over. It can be explained on the basis of contemporaneous conception of brain oedema, which postulates short-term and temporary opening of BBB to be sufficient for induction "tissue damage", with subsequently developing cellular component of the brain's internal environment changes (Go 1997).

Our observation and results confirm experience of other authors (see above) who assumed brain oedema to be an initialising factor of axonal changes. The specific sensitivity of axons to the microenvironmental changes in the brain may reflect the level of plasticity and local specialization of microdomains of the neuronal membrane (Mourek et al. 2009). Experimental conditions enabled us to prove that axonal impairment developed very early after the oedema induction (one hour) and it progressed during the following days. Conversely, no spontaneous recovery of axonal changes was identified, at least within the first week after the stimulus. Though the histological image indicated subsequent progression of axonal changes, no behavioural changes or obvious neurological deterioration were observed in our experimental animals. Important is therefore the proportion of impaired and intact axons and the location of injured ones in the brain (see also Gennarelli 1998).

CONCLUSION
Opening the BBB with an osmotic insult (20% mannitol applied into the internal carotid) induces brain oedema which represents a factor triggering myelin changes in the studied areas of the hippocampus and cerebral cortex in both hemispheres. Such development of axonal changes initiated only by brain oedema (without ischemia brain injury) has not yet been described and any explanation is thus hypothetical. The progression of myelin damage depends on the time elapsed after the BBB opening: the lowest grade develops in one hour, the highest grade requires one week. All experimental animals survived, showed standard behaviour and were free from signs of neurological deficit.

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