Effects of Intracarotid Injection of Methylprednisolone on Cellular Oedema after Osmotic Opening of the Blood-Brain Barrier in Rats

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Dedicated to professor MUDr. Stanislav Trojan, DrSc. to the 70th birthday

Abstract: In our work we studied methylprednisolone (MP) for its effects on the permeability of cytoplasmatic membranes of neuronal populations in the rat. We used a standard model of cellular oedema induced by water intoxication, applying MP selectively into the internal carotid (ICA) after opening the blood-brain-barrier (BBB) with mannitol. The results were assessed under fluorescence microscopy in keeping with the Intracellular Distribution Index of Evans Blue (IDI) in the neocortical field (Cortex) and in hippocampal areas CA1, CA3 and GD. Evans blue (EB) was applied similarly as MP. Three different experiments were carried out. In experiment 1 – EB alone and no MP was applied. In experiment 2 – 5.4 mg/kg MP and EB were applied. In experiment 3 – 54 mg/kg MP and EB were applied. In experiment 1 the IDI values were high (>1), indicating the presence of large quantities of EB in the cells. In experiments 2 and 3 the IDI values were low (<1), indicating more EB outside than inside cells. IDI differences between experiments 2 and 1 and experiments 3 and 1 were statistically significant ($p<0.05$). This morphological evidence sufficiently proved the possibility to restore the cell membrane integrity by means of MP administration.

Key words: Cellular oedema – Methylprednisolone – Blood-brain-barrier – Intracarotid injection – Fluorescence microscopy

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Introduction

Neuroprotective drugs are a subject of unflagging interest in experimental projects and clinical trials [1]. So far the only neuroprotective substance known to influence the natural course of pathophysiological conditions after severe CNS injury has been found in methylprednisolone, the positive effect of which was shown in a randomised double-blind study [2]. The primary neuroprotective effect of methylprednisolone (MP) is put down to its antioxidative capacity for removing lipid peroxides in cell membrane. The process of antioxidation protects cell membranes against damage mediated by free radicals released by unsaturated fatty acids oxidation going on in the cells in the presence of molecular oxygen. Hence, the main neuroprotective effect of MP rests in that it forestalls irreversible lipid peroxidation and a cascade of events resulting from a shortage of sources of energy. This takes the form of a change in the flow of ions on the cell membrane leading to intracellular oedema and accumulation of extracellular glutamate, which is released in considerable quantities from postsynaptic membranes. Activated glutamate receptors open calcium channels and set off calcium influx into the cell. High levels of intracellular calcium cause damage to the cytoskeleton beside activating phospholipase A. That, in turn, sets off a cascade of arachidonic acid metabolites while released toxic eicosanoids (prostaglandins, leucotriens and thromboxanes) induce aggregation of thrombocytes, vasoconstriction and intravascular thrombosis. In another effect, arachidonic acid metabolites release more lipid peroxides, thus completing membrane damage [3, 4, 2, 5, 6, 7]. Methylprednisolone's antioxidative ability to avert lipid peroxidation can only be exploited if the drug is given in a large dose (30 mg/kg i.v.) as soon as possible after injury (within three hours) with subsequent continual 24-hour application (5.4 mg/kg/hour). This procedure resulted from three randomised double-blind studies taking place at many centres during the 1979–1998 period under the name of NASCIS (National Acute Spinal Cord Injury Study) I, II, III, regarded as standard method for the treatment of spinal cord injury [8, 9, 10]. Neuroprotective effects of methylprednisolone have failed to be proved in brain injuries, and corticotherapy is not recommended there [11, 12].

This difference in the effects of the same substance (methylprednisolone) on posttraumatic situation in different portions of the CNS (brain and spinal cord) has yet to be reliably explained. The spectrum of experimental models designed to test the neuroprotective effects of MP in the brain consists mainly of diffuse brain injury, brain oedema and ischaemia. In those models, MP is applied intravenously, intraperitoneally or intraarterially in different doses, and the parameters designed to test the effects of MP differ from one another [13, 2, 14, 15, 16, 17, 18]. We applied MP selectively into the internal carotid after opening the BBB with mannitol [19, 20, 21, 22]. In our view, the intracarotid method offers a number of advantages. The first one is in that using the method of osmotic insult to open the blood-brain-barrier it is possible to convey into the brain even those

substances, which under physiological conditions fail to pass through the BBB. Second, the amount of the active substance applied by way of the carotid, i.e., right into the brain, can be substantially less than that required for the application of the same substance into the systemic vascular bed. And last, there is the significant fact that the substance applied into the carotid will soon reach a high intracerebral concentration and that its effect will set in faster. Selective active substance application into the internal carotid is a therapeutical method currently used mainly in neurooncology [23, 24, 19]. The neuroprotective effects of MP applied selectively into the internal carotid after BBB opening with mannitol was tested on a standard model of cellular oedema induced by water intoxication (hyperhydration) [25, 26, 27, 28, 29, 30]. Intoxication with water and hypo-osmolality are always associated with hyponatremia, which is why current natremia is an important control value [28].

Material and methods

Our experimental work took place in the Neurohistological laboratory of the Institute of Physiology, 1st Faculty of Medicine, Charles University, Prague. Adult Wistar strain laboratory rats of both sexes were used for the purpose. The animals' weight ranged between 350 and 450 g. Animals were treated in accordance with valid guidelines for work with laboratory animals (EU Guidelines 86/609/EEC). Before the experiment, animals were hyperhydrated with the method of water intoxication [25, 26, 27, 28]. Each was given distilled water in a quantity equal to 20% of the body weight in three separate doses applied intraperitoneally at 8-hour intervals during 24 hours prior to launching the experiment. All had their BBB opened by mannitol. General anaesthesia was induced with an i.p. injection of thiopental 4 mg/100 g with the animal breathing spontaneously throughout the procedure. After a skin incision was made along the midline between the upper edge of the sternum and the mandible, standard microsurgical technique was used to expose the right ACC (arteria carotis communis) and, behind its bifurcation, the proximal parts of the ACI (arteria carotis interna) and ACE (arteria carotis externa), which was ligated close beyond the bifurcation (Colour Fig. 6). An intraluminar catheter was introduced from an arteriotomy into the ACC trunk for 20% mannitol (200 g ad 1000 ml aqua pro iniectione, 1098 mosmol/l) to be infused selectively into the internal carotid at a dose of 5 ml/kg and at a rate of 0.12 ml/min [31]. After BBB opening (30 seconds after mannitol infusion) MP and then 2% Evans blue (dose 2ml/kg) at a rate of 0.45 ml/min [32] were injected into the same catheter. The MP dose differed by the type of each particular experiment. With the application over and the catheter removed, the ACC was ligated distal and proximal to the arteriotomy. Since the laboratory rat has a fully developed Willis circuit, obliteration of one of the extracerebral arteries can hardly have any major effect on the intracerebral distribution of the substance or the marker [33]. After wound suturing the spontaneously ventilating animal was placed in a heat-insulated box for a period of 30 minutes. After that, fixation was performed by

transcardial perfusion with 4% solution of paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 15 minutes, whereupon the brain was left immersed in the same solution for 24 hours. Then, using a vibratome, coronary sections 40 μm thick were obtained. Without any further staining they placed on glass slides, dehydrated and covered with DePex for fluorescence microscopy (OLYMPUS-Provis) analysis.

Three experiments were done, differing as to MP dosage. The results were assessed in five laboratory rats per each experiment. Excluded from the series were all animals, in which technical problems arose during the operation or which developed ventilation disorders by the start of infusion. Similarly, left aside were three rats hyperhydrated in the standard way after they had blood samples taken from the ACC with an intraluminar catheter for current natremia estimation.

In experiment 1, EB alone and no MP were applied. In experiment 2, we applied 5.4 mg/kg MP and then EB in quick succession. In experiment 3, we applied a dose of 54 mg/kg MP and EB immediately afterwards.

Neurohistological image analysis yielded information on EB distribution in the extracellular and intracellular compartments in each particular experiment.

Under experimental conditions, mannitol-altered BBB permeability is tested by a number of markers. Today's most frequently used marker – Evans blue (EB, MW 961) – passes through the BBB on intracarotid application as a pure dyestuff unlike application into the systemic vascular bed where it bonds to the albumin fraction of plasma proteins to produce a high-molecular marker EBA (MW 68 500) [24, 34, 35, 36]. In our experiments, we applied EB (MW 961) selectively into the internal carotid after osmotic opening of the BBB, to register its distribution in the brain depending on the type of experiment. As shown in our previous works, for purposes of standardisation and results interpretation it was expedient to study in detail only predetermined areas [20, 21, 22]. These were, in particular, a neocortical temporal field (Cortex) on the level of the hippocampus (3.0–3.5 mm posterior to bregma) and hippocampal formation divided into areas CA1, CA3 and hilus of the dentate gyrus (GD). Each was studied separately in the ipsilateral (right) and contralateral (left) hemispheres. Assessment of EB distribution in the regions under study was based on the hypothesis that all of the fluorescence extravasate can be divided into only two compartments, either retaining its extracellular location or, in addition, penetrating into specific cell populations. EB contained in cells is then missing from the extracellular compartment so that its distribution can be described as extracellular or intracellular. It has to be emphasised, though, that the term intracellular is understood to mean intraneuronal in areas with strictly deployed neuronal populations (CA1, CA3, GD) while in areas where this stratification is not quite so prominent (Cortex) we refer to intraneuronal and intraglial distribution. As follows from our previous studies, EB distribution in the areas under scrutiny corresponds to the intensity of emitted fluorescence, which can be registered under fluorescence microscopy in cells as well as in the extracellular compartment. Poor quantifiability is a disadvantage of registering the

emitted fluorescence intensity. For that purpose, we resorted to the "LUCIA image analysis system" computer software to transpose the fluorescence into a black-and-white scale of different degrees of grey ranging from 0 to 256 points, where 0 stands for the lightest point, and 256 for the darkest point on the scale. Using the set of points obtained for each area explored, we established the intracellular distribution index (IDI) according to the equation: IDI = L × 0.0078125 where IDI = intracellular distribution index, L = point on the 0–256 scale, and 0.0078125 = coefficient (2:256 = 0.0078125). IDI is a precisely defined quantity from 0 to 2 where, with IDI = 1, the quantity of EB in the cells is equal to that in the extracellular compartment. Using the above method, we established the IDI in five rats with a standard course of each experiment. The IDI value thus obtained represented the statistical average of five computer-processed morphological analyses for each area under study. In our model, the MP effect took the form of altered EB distribution expressed as the IDI value in experiments 2 and 3 as distinct from experiment 1. All results were analysed for statistical significance using a two-tailed Student *t* test.

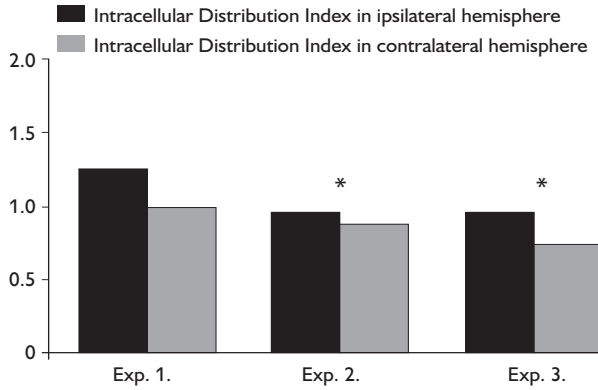

Fig. 1 – CORTEX
MP reduced significantly
(p<0,05) IDI in Exp. 2. and 3. (*)

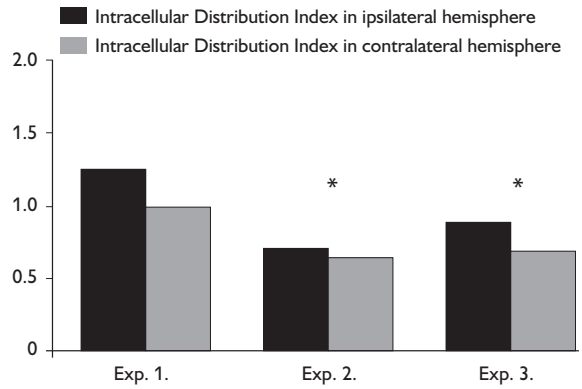

Fig. 2 – CA1
MP reduced significantly
(p<0,05) IDI in Exp. 2. and 3. (*)

Results

Physiological natremia in laboratory rodents (mice, rats) ranges from 143 to 156 mmol/l, with hyponatremia having values at least 20 mmol/l lower than the above normal range. Hyponatremia less than 120 mmol/l represents very reliable indicator of hyperhydration-induced cellular oedema [28]. Current natremia was assayed in three standard hyperhydrated control animals. The values of 112.6 mmol/l, 97.8 mmol/l and 114.8 mmol/l actually found there clearly show the method of hyperhydration used in all our experiments as leading reliably to cellular oedema.

The results in experiments 1 – 3 were rated according to the IDI value in each of the areas explored in both hemispheres. With IDI = 1, the distribution of EB between the intracellular and extracellular compartments is in equilibrium. IDI in excess of 1 indicates more EB in the cells than in the extracellular compartment of the particular area. When IDI is less than 1, most of the EB remains outside cells

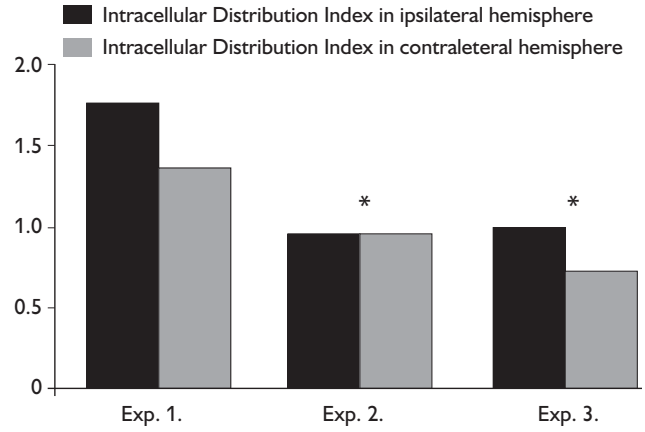

Fig. 3 – CA3
MP reduced significantly
(p<0,05) IDI in Exp. 2. and 3. (*)

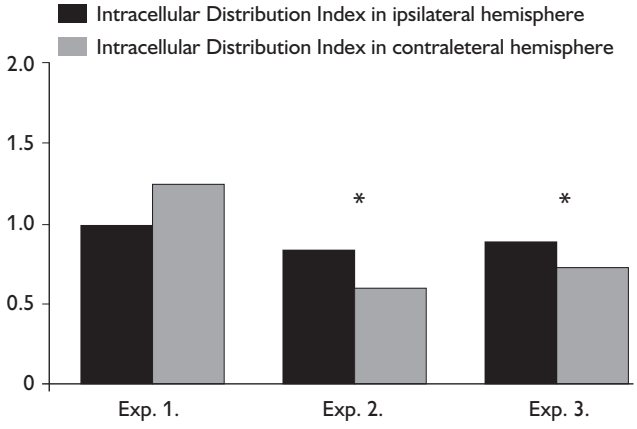

Fig. 4 – GD
MP reduced significantly
(p<0,05) IDI in Exp. 2. and 3. (*)

with only its lesser portion penetrating the cells (Colour Fig. 4 a, b and 5 a, b). The IDI values obtained in experiments 1 – 3 are graphically represented separately for the Cortex (Fig. 1), for CA1 (Fig. 2), CA3 (Fig. 3) and for GD (Fig. 4). The graphs clearly show that in experiment 1 the IDI values are higher than 1, and in experiments 2 and 3 the IDI values are lower than 1. All results were analysed for statistical significance using a two-tailed Student *t* test. Statistically significant differences were found between experiments 2 and 1 and between experiments 3 and 1 (Tab. 1 and 2).

Discussion

Methylprednisolone, a synthetic steroid, has four times higher glucotropic effect and five times lower mineralotropic effect than cortisol (hydrocortisone). As a pure steroid, MP is lipophilic but poorly soluble in water. For easier distribution in all body fluids it has to be applied in the form of the ester MPSS (methylprednisolone sodium succinate). This compound, however, lacks stability; MP is released from it under the effect of hepatic esterases, and, while in

Table 1 – Two-tailed *t*-test for comparison of Experiments 1 and 2.

	DF	T value	p
ipsi C	8	6.15	0.0003
ipsi CA1	8	11.11	< 0.0001
ipsi CA3	8	14.86	< 0.0001
ipsi GD	8	6.06	0.0003
contra C	8	5.67	0.0005
contra CA1	8	6.04	0.0003
contra CA3	8	8.69	< 0.0001
contra GD	8	8.78	< 0.0001

ipsi – ipsilateral hemisphere; contra – contralateral hemisphere; C – cortex; CA1, CA3, GD – areas of hippocampus; DF – degree of freedom

Table 2 – Two-tailed *t*-test for comparison of Experiments 1 and 3.

	DF	T value	p
ipsi C	8	6.78	0.0001
ipsi CA1	8	5.03	0.0010
ipsi CA3	8	7.73	< 0.0001
ipsi GD	8	5.06	0.0010
contra C	8	6.22	0.0003
contra CA1	8	8.84	< 0.0001
contra CA3	8	9.55	< 0.0001
contra GD	8	7.12	< 0.0001

ipsi – ipsilateral hemisphere; contra – contralateral hemisphere; C – cortex; CA1, CA3, GD – areas of hippocampus; DF – degree of freedom

circulation, some 40–60% of it is bonded to plasma proteins. This bond gives rise to a high-molecular substance which cannot pass through the BBB. Only pure liposoluble MP can do that. Out of the total amount of MPSS applied i.v. or i.p. only about one half will cross the BBB [2].

As follows from many experiments designed to study the neuroprotective effects of MP in spinal cord trauma, lipid peroxidation is inhibited at a 100 μmol MP concentration in the plasma membrane [37]. Apart from the primary antioxidative effect, these experiments helped to discover some other neuroprotective effects of MP as well. These include blood circulation control in nervous tissue [38, 39], aerobic metabolism control [40, 41] and neuronal excitability and synaptic transmission enhancement [42]. Thanks to those properties and to its primary antioxidative effect, MP is believed to act as a cell membrane stabiliser [3]. According to Hall, experimental evidence of the MP neuroprotective influence in spinal cord trauma could also be exploited in experimental injury of the brain. Admittedly, he adds, such experiments – compared with spinal cord injuries – are substantially fewer, their complete analysis is missing, and the results published so far are not unambiguous [2]. As regards brain injury, the neuroprotective effect of MP was demonstrated in some experiments [43, 17, 13] while in others it was not [16]. Vasogenic oedema reduction in response to MP was documented by Lin et al. [15]. The drug's neuroprotective effect was proved also in some experimental models of brain ischaemia [44, 18]. The overall quantity of MP differs from author to author (30 mg/kg up to 105 mg/kg) as do the dosage (at least one, at most five doses) and time of infusion (a few minutes up to 12 hours). This shows lack of uniformity in experimental testing for MP neuroprotective effects on the brain, thus bringing out the fact that its primary effect on brain cells has yet to be fully elucidated; for the time being, we have to define MP as a cell membrane stabiliser [2, 3]. We believe that beside the overall quantity and MP dosage at particular intervals of time, making good use of its neuroprotective properties may be influenced by the mode of application. In the experiments so far, MP was applied mainly intravenously or intraperitoneally and, in one case, intraarterially by way of the caudal artery. For MP to act on the target structure – brain cell membrane – it would have to permeate the BBB regardless of the actual mode of application. As mentioned above, only about half the dose applied will pass through the BBB. The above quoted studies make no mention of this important phenomenon.

In our own experiments we applied MP selectively into the internal carotid having opened the BBB with mannitol in laboratory rats with induced cellular oedema. This procedure makes use of the experimental model of "blood-brain barrier manipulation" developed in our laboratory for studies of the brain's internal environment [20, 21, 22]. Essentially, it is based on a neurohistological picture of EB distribution in the intracellular and extracellular compartments of the areas

under study given different states of the BBB (intact or opened with mannitol) and neuronal formations (normal or hyperhydrated animals). The neurohistological picture of EB distribution is constant for each situation explored. Hence, changes in the brain's internal environment are easy to register and reproduce. MP infused selectively into the internal carotid after opening the BBB with mannitol permeated into the brain as a pure steroid because it does not bond to plasma proteins similarly as EB applied in the same way will not create an EBA complex. Presumably, this procedure helps to exploit the neuroprotective properties of the whole dose of MP.

In our experiments, the neurohistological image of EB distribution expressed as the IDI value in the water intoxicated laboratory rat was compared with altered EB distribution found in an equally hyperhydrated animal after MP infusion. The MP effect was tested with respect to a variously large one-off dose (5.4 mg/kg or 54 mg/kg). Using the dose of 5.4 mg/kg we wanted to verify some authors' claim that the substance applied into the carotid after BBB opening is efficacious in much smaller quantities than if it is applied into the systemic vascular bed [19, 24]. The specific dose of 5.4 mg/kg was chosen because it now represents the smallest amount of MP used for neuroprotective purposes in clinical practice (10). The other dose (54 mg/kg) was chosen because it is equal to roughly one half of the largest dose of MP (105 mg/kg) as yet used in testing its neuroprotective effects in an experimental model with intraarterial application [18]. A dose of 54 mg/kg applied into the carotid ought to have a neuroprotective effect comparable to 105 mg/kg applied into a peripheral artery [2]. The IDI is a constant parameter enabling us to compare neurohistological findings in our experiments. With the IDI equal to 1, intracellular and extracellular EB distributions are the same. IDI greater than 1 indicates that EB is present more inside than outside cells while with IDI less than 1 it is the other way round. In experiment 1, only EB was applied and no MP. In the overwhelming majority of the areas under study, IDI is greater than 1 (Fig. 1–4) in what is morphological proof of the water-soluble low-molecular intravital marker (EB, MW = 961) permeation of the cell membrane. Increased permeability reflects loss of membrane integrity. The primary role in this phenomenon is played by inhibition of the physiological function of membrane Na+/K+ ATPase as a result of disordered cellular-level osmoregulation due to water intoxication [45, 46, 47, 30, 29]. In experiments 2 and 3, we applied EB immediately after the application of the tested dose of MP (5.4 mg/kg and 54 mg/kg respectively). The neurohistological results in those experiments were found differing significantly from those in experiment 1 (Tab. 1 and 2). In all the areas under study the IDI is less than 1,which means that inside the cells there is less EB than in the extracellular compartment (Fig. 1–4). In our view, this is morphological evidence of cell membrane integrity restored under the effect of MP. Since our experiments are based on morphological studies, rather than speculate about the

relevance of the different characteristics of MP we can only weigh its comprehensive, stabilising effect on cell membranes [2, 3]. However, we do dare hypothesise that MP intervenes while still in the phase of exploitability of its antioxidative properties, thus forestalling irreversible lipid peroxidation and creating the conditions for preserving cell membrane functions [3, 4, 2, 5, 6, 7]. In experiments 2 and 3 we found no statistically significant difference in IDI values between the MP dosages of 5.4 mg/kg and 54 mg/kg. As these results show, even a small dose of the tested drug infused selectively into the internal carotid, with the BBB open, will instantly reach efficacious concentration in the brain [19, 24].

In conclusion our morphological study shows that MP in doses of 5.4 mg/kg or 54 mg/kg applied into the internal carotid after BBB opening with mannitol brings a significant reduction of cytoplasmic membrane permeability in a model of cellular oedema induced by water intoxication in the rat.

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