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"COMPENSATED HYPEROSMOLARITY" OF CEREBROSPINAL FLUID AND THE DEVELOPMENT OF HYDROCEPHALUS

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Abbreviations: CM - cisterna magna; CP - choroid plexus; CSF - cerebrospinal fluid; ICP - intracranial pressure; LLV - left lateral ventricle; RLV - right lateral ventricle; SAS - subarachnoid space; SD – standard deviation.

Abstract

Acute osmolar loading of cerebrospinal fluid within one lateral ventricle of dogs was examined as a cause of water extraction from the bloodstream and an increase in intracranial pressure. We have shown that a certain amount of $^{3}H_2O$ from the bloodstream enters osmotically loaded cerebrospinal fluid significantly faster, hence causing a significant increase in intracranial pressure. The noted phenomenon in which intracranial pressure still significantly increases, but in which the hyperosmolarity of the cerebrospinal fluid is no longer present, was named "compensated hyperosmolarity". In the case of the sub-chronic application of hyperosmolar solutions into cat ventricles, we observed an increase in cerebrospinal fluid volume and a more pronounced development of hydrocephalus in the area of application, but without significant increase in intracranial pressure and without blockage of cerebrospinal fluid pathways. These results support the newly proposed hypothesis of cerebrospinal fluid hydrodynamics and the ability to develop new strategies for the treatment of cerebrospinal fluid-related diseases.

Keywords: cerebrospinal fluid; hydrocephalus; cerebrospinal fluid hydrodynamics; cerebrospinal fluid hyperosmolarity; intracranial pressure

INTRODUCTION

For nearly a hundred years, the so-called "classical" hypothesis of the physiology of cerebrospinal fluid (CSF) has persisted. According to this hypothesis, CSF physiology is based on three key premises: active CSF formation (secretion); passive absorption of CSF; and the unidirectional flow of CSF from the site of formation to the site of absorption (circulation). It is assumed that the main production sites of CSF (70-80%) are the choroid plexuses (CPes) inside the brain ventricles, which is where filtration across the endothelial capillary wall and secretion through the choroidal epithelium occur. The remaining 20-30 % of CSF production occurs as a bulk flow of interstitial fluid, probably produced by the ependyma (O'Connel, 1970; Milchorat, 1972; Pollay, 1975; McComb, 1983; Davson *et al*., 1987; Cserr, 1989; Brown *et al*., 2004; Johanson *et al*., 2008). According to this view, CSF is formed actively by the secretory activity of the CPes, structures which represent "CSF pumps" (Bering, 1955; Orešković and Klarica, 2011) and which are positioned exclusively inside the brain ventricles (Fig. 1a).

It is generally accepted that CSF, as a slow river, circulates unidirectionally from the brain ventricles (the place of CSF formation) into the cortical subarachnoid space (SAS; the place of CSF absorption). It is also believed that CSF circulation results from the pumping pulsatile action of the CPes. The pulsation of CSF is generated mainly by the filling and draining of the CPes. Each pulse should set up a pressure gradient throughout the CSF system which forces CSF out of the cerebral ventricles. This way, the CPes act as an unvalved pulsatile CSF pump, imparting a constant to-and-fro CSF motion (Bering, 1955). Furthermore, it is believed that a constant to-and-fro CSF motion could be also generated by the distensibility of the spinal dura (Martins et al., 1972) and the compressibility and emptying of the veins in spinal and cranial cavities (Foltz, 1994). NM imaging shows that, during systolic vessels expansion, an almost simultaneous craniocaudal CSF displacement occurs in the aqueduct of Sylvius, the basal

cistern and the cervical subarachnoid space, while during diastole, the caudocranial dislocation of CSF appears due to a decrease in brain blood volume and the recoil of displaced CSF in the spinal region (Alperin et al., 2005; Enzman and Pelc, 1991; Feinberg and Mark, 1987; Greitz et al., 1993).

The arachnoid villi inside the dural venous sinuses have generally been thought to be the main site of CSF absorption. It is believed that CSF is passively absorbed from the cortical CSF into the cranial venous blood by means of a hydrostatic gradient (Weed, 1935; Brodbelt and Stoodley, 2007; Pollay, 2010). Recently, by experiments in vitro demonstrated on archnoid granulations cells some investigators (Holman et al., 2005; Grzybowski et al., 2006) trying to present arachnoid villi in vivo as a place that regulate CSF absorption. In addition, there is a large amount of literature which suggests that the significant absorption of CSF occurs from the subarachnoid space to the lymphatic system (Dandy, 1929; Brierly and Field, 1948; Bradbury, 1981; Johnston et al., 2005; Koh et al., 2006). With minor modifications, these are the main postulates of the classical hypothesis. It should also be added that the total CSF volume is a result of the ongoing relationship between active CSF formation through “CSF pumps” and passive CSF absorption. This means that in physiological conditions, the same CSF volume which is actively formed within the brain ventricles must be passively absorbed from the cortical SAS (Orešković and Klarica, 2010). Today, this hypothesis still represents a common point of reference in scientific papers, review articles and numerous textbooks, and is presented as a fact.

The hypothesis is also applied as a key explanatory factor in the popular (circulation) theory of the hydrocephalus development. It is assumed that hydrocephalus is a result of a discrepancy between CSF secretion and absorption, with a subsequent CSF accumulation in the cranial cavity and its enlargement (mainly in the brain ventricles). The balance between the secretion and absorption of CSF is critically important. Because CSF is produced

continuously by “CSF pumps” pushing against increased CSF/intracranial pressure (ICP; Pollay *et al.,* 1983; Davson *et al.,* 1987), medical conditions that block its normal circulation or absorption will result in a CSF over-accumulation in front of the blockade. In other words, if the CSF system is obstructed between the place of CSF secretion and the place of its absorption, the CSF cranial space should, because of the continuity of CSF secretion through the “CSF pumps”, dilate and produce hydrocephalus.

Recently, the “classical” CSF hypothesis has been increasingly challenged and a new hypothesis regarding CSF physiology has been proposed (Klarica *et al.,* 2009; Orešković and Klarica, 2010; Bulat and Klarica, 2011; Orešković and Klarica, 2011). According to this new hypothesis (Fig. 1b), CSF is not secreted mainly inside brain ventricles and does not subsequently circulate unidirectionally to finally be absorbed outside of ventricles, but freely exchange throughout the entire CSF system, depending on the hydrostatic and osmotic forces between the CSF, interstitial fluid (ISF) and blood capillaries. Osmotic and hydrostatic forces are crucial to the regulation of ISF-CSF volume, which represents a functional unit (Orešković and Klarica; 2010; 2011). In terms of the capacity of fluid volume exchange, the cerebral capillaries are the dominant location, and the CPes are a less relevant site for this process. It has been estimated that the capillary-brain surface area is about 5000 times greater than the choroidal area (Coulter, 1958; Wald *et al.,* 1976; Klarica, M. *et al.,* 2009).

There is permanent fluid and substance exchange between the CSF system and the surrounding tissue, which depends on the (patho)physiological conditions that predominate within these compartments. It was shown by Rennels et al. (1985) that, after horseradish peroxidase (HRP; m.w. 40,000) was infused into a lateral ventricle or cisterna magna of cats and dogs for 4-10 min under normal CSF pressure, it was distributed in the perivascular (Virchow-Robin) spaces of the whole brain parenchyma so that all the blood vessels, including capillaries, were outlined. This rapid perivascular distribution of HRP in the brain

can be prevented by stopping or diminishing the pulsations of cerebral arteries by aortic occlusion or by partial ligation of the brachiocephalic artery (Rennels et al., 1985). Thus, the pulse pressure in CSF and the parenchyma (DiRocco, 1984) should be instrumental in the rapid perivascular distribution of substances from CSF but not in a slow diffusional process (Rennels et al., 1985). A similar phenomenon was described by Iliff et all. (Iliff et all 2012; Iliff et all. 2013) on anaesthetized mice using modern neuroimaging tecniques for monitoring the fate of different molecules inside the CSF and ISF (fluorescent tracers or paramagnetic contrast with different m.w.; soluble amyloid β). After the application of tracer molecules into ventricles and cisterna magna a fast distribution along the adjecent cranial CSF spaces, and from there into the interstitium along the paravascular spaces was noticed (initially along the penetrating arteries and subsequently along paravenous pathway). Zmajević at al., (2002) have shown in more physiological condition on free moving cats the way in which individual molecules (dye phenosulphophtalein which imitates the fate of cerebral metabolites) applied into CSF are being disposed of by reaching the tissue, and by means of active transport at the capillaries leave the CNS. Blockade of active transport at the BBB level prolonged the stay of the dye inside the tissue and the CSF, and thus led to the wider distribution along the CSF system as well as the brain and spinal cord tissue. A significant distribution from the cisterna magna to the spinal space and tissue as well as to the lateral ventricle also goes against the classical concept of the unidirectional CSF circulation, according to which substances should in time be carried mostly to the cortical subarachnoid space from which the CSF is absorbed (Zmajević et al 2002; Vladić et al. 2009).

In other words, there is no special place within the CSF system exclusively for CSF formation or CSF absorption. In light of new hypothesis, the accepted circulation concept regarding hydrocephalus development has also been re-evaluated and a new one has been proposed (Orešković and Klarica, 2011).

The fact is that hydrocephalus is an excessive amount of CSF which accumulates in the cranium within or outside the brain. Since 99% of CSF is water (Bulat *et al.,* 2008), it is evident that hydrocephalus is an excessive amount of water. As there are almost no obstacles/barriers for water within the CNS, and as water quickly and easily crosses from one compartment to another (blood, CSF, ISF, intracellular fluid), the cause of excessive fluid accumulation should be searched for in pathophysiological conditions leading to the displacement of water into the CSF space, and its accumulation within the CSF system. It is well documented that osmotic gradients play a significant part in the regulation of brain water and CSF volume (Hochwald *et al.,* 1974; Wald *et al.,* 1976; Orešković *et al.,* 2002; Maraković *et al.,* 2010; Jurjević *et al.,* 2012). Because of this, it has been presumed that without significant obstruction or stenosis of the CSF system, all pathological processes in which an increase of CSF osmolarity (the osmotic load of CSF) takes place should lead to an increase in CSF volume, and consequently should cause hydrocephalus (Krishnamurthy *et al.,* 2009; Orešković and Klarica 2010; 2011) what speaks in favor the most recent multiinstitutional studies of hydrocephalus as a consequence of hemispherectomy surgery for medically intractable epilepsy treatment (Lew et al., 2013).

On the basis of the above and assumptions that the cerebral capillaries are the dominant location of fluid volume exchange capacity, it is of the utmost importance to examine how water from the bloodstream behaves when CSF is osmotically loaded. For this purpose, an experimental model in dogs was developed (Fig. 2a; see Methods), where acute hyperosmolar CSF is experimentally produced in only the right lateral brain ventricle (RLV; Table 1). This way, we achieved a very sensitive model by means of which water influx from the bloodstream into both ventricular hyperosmolar CSF and also the iso-osmolar part of the CSF was possible (Klarica *et al.,* 1994). This access is of crucial importance in obtaining basic answers related to the interaction of CSF hyperosmolarity and water from the bloodstream as

a possible reason for CSF volume accumulation and cause of ICP increase. Using this model, we also observed and described the phenomenon of the "compensated hyperosmolarity" of CSF for the first time. The term "compensated hyperosmolarity" represents the time period when CSF hyperosmolarity has already disappeared but the effect of hyperosmolarity still persists in the CSF system. Since we assume that hydrocephalus often develops over a prolonged period (Orešković and Klarica, 2011), and the fact that hydrocephalus was experimentally produced in rats by means of chronic hyperosmolar infusion (Krishnamurthy *et al.,* 2009), the development of hydrocephalus was studied in cats through subchronic micro-volume infusion of hyperosmolar solution into the right lateral ventricle (RLV). Furthermore, if the blockade of the CSF system is not essential for the development of hydrocephalus, it should be expected that the development of hydrocephalus begins at the infusion site and then spreads through the rest of the CSF system.

EXPERIMENTAL PROCEDURES

The experiments were performed on 23 adult dogs and 8 cats, unselected for age and sex, ranging in weight from 11.0 to 25.0 kg for dogs and 1.6 to 3.8 kg for cats. All experimental procedures were performed in accordance with the European Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes, the Law on Animal Rights and Protection of the Republic of Croatia and with the approval of the Ethical Committee of the University of Zagreb, School of Medicine (No: 380-59/12-302/60).

Experiments were performed on mongrel dogs anesthetized with α-chloralose (100 mg/kg; i.p.) and with their head fixed in a stereotaxic frame micromanipulator into both lateral ventricles at coordinates 5-6 mm anteriorly and 7-8 mm laterally (left and right) from the zero point of the stereotaxic atlas depending on the dogs weight, and 12-15 mm vertically from dural surfaces, until free communication with CSF in the ventricle was obtained (Klarica *et al.,* 1994). An additional cannula was positioned into the RLV, 2 mm anteriorly to that previously positioned which served for CSF pressure recording *via* a pressure transducer (Gould P 23 ID, USA) and a polygraph (Grass, Model 7, USA). A pressure transducer was calibrated at the level of the inter-auricular line using a water column. ICP is presented as cm H_2O. All of these cannulas were fixed with dental cement to the skull. The cisterna magna was punctured with a cannula (Fig. 2a). The femoral artery was cannulated for blood sampling to determinate the ^3H_2O concentration and acid-base balance. No significant changes in blood pressure or blood gases were observed in these experiments on dogs with spontaneous breathing under chloralose anaesthesia. ^{3}H-water (spec. act. 1 mCi/g, New England Nuclear, USA) standard solution was prepared (50 μL ^3H_2O 1 mCi/g H_2O in 12 mL of saline) and the femoral vein was cannulated for the application of 4 mL ^3H_2O standard solution (9 x 10^6 dpm/50 μL) in bolus during 5 seconds.

Acute application of sucrose solution (950 mmol/L H_2O) was performed by means of the micro-volume exchange method (Fig. 2a). A sucrose solution of 950 mmol/L H_2O was chosen, because this was the osmolarity which was sufficient to cause a significant effect on CSF pressure (Fig. 2b) 5 minutes after application. It was performed in the following manner: 100 µL of CSF was withdrawn from the cisterna magna and the same volume of sucrose was applied into the right lateral ventricle or cortical SAS. This procedure was repeated ten times over 1 minute until the desired solution volume (1 mL) was applied. ICP was continuously recorded before, during and after the application, and only small ICP changes could be observed during the micro-volume exchange of the solutions applied into the CSF (Klarica *et al.,* 1994). Thus, we want to emphasize that the saline applicationin into the CSF system (RLV) under the same conditions (micro-volume exchange method) did not considerably change either CSF volume or ICP (Klarica *et al.,* 1994).

In Table 1, we have shown the osmolarity spread tested on this model in different time intervals in the left (LLV) and right lateral ventricles (RLV), and in the cisterna magna (CM). It can be seen that the application of hyperosmolar solution after five minutes resulted in a significant increase in CSF osmolarity in the RLV and remained significantly higher over 35 minutes. Simultaneously measured osmolarity in other parts of the CSF system (left lateral ventricle and cisterna magna) did not show any increase in osmolarity. This shows that hyperosmolar CSF did not significantly spread outside the right lateral ventricles. Fifty minutes after the hyperosmolar sucrose application, CSF was iso-osmolar in all measured CSF areas. The solutions osmolarity was analyzed by means of an osmometer with a freezing point depression (Knauer, Germany).

Arterial blood samples (0.5 mL) were obtained from the cannulated femoral artery. The samples of ventricular or cisternal CSF (50 µL) were obtained through the cannulas positioned in the lateral ventricles or cisterna magna with a 25 gauge cannula which was fixed

in position by a holder. It is interesting to note that we have always been able to collect CSF samples from the right (hyperosmolar) lateral ventricle, unlike the sampling from the left (iso-osmolar) lateral ventricle. The samples were taken in the first, in the third, in the eighth, in the twentieth and thirty fifth minute after ^3H_2O application (Fig. 3a). The radioactivity of CSF (50 µL) and arterial plasma samples was measured in 2 mL of scintillation solution (HP-Ready Solv, Beckman) by a liquid scintillation counter (Beckman LS 1701, USA).

The sub-chronic application of sucrose solution in freely moving cats was done by osmotic mini-pump slow infusion over 7 days into the CSF of the lateral ventricle. The cats were anaesthetized with thiopenton sodium (50 mg/kg i.p). The head of the animal was fixed in a stereotaxic frame (D. Kopf, USA). The vertical level of the external auditory meatus was taken as the zero level. For the substances infusion into the lateral ventricle, a drill hole (1.5 mm i. d.) in the parietal bone, 2.5 mm lateral of the mid-sagittal line and 8 mm caudal from the coronal suture was performed and the dura exposed. A stainless-steel cannula (25 gauge) was introduced by means of a micromanipulator into the lateral ventricle (10–12 mm below the dura level) and the external end of the cannula was connected by means of plastic tubing to an osmotic mini-pump (model 2ML1, Alza Corp., USA). The mini-pump and connection tubing had previously been filled with hyperosmolar sucrose (2400 mmol/L H_2O). The osmotic mini-pump has a mean fill volume of 2117 ± 55 (SD) µL and ejects the solution at a mean pumping rate of 10.12 ± 0.41 (SD) µL per hour, i.e. about 0.024 mmol per hour. The osmolarity of the sucrose used in the mini-pump was assorted in such a manner as to cause a small but significant elevation in CSF osmolarity after being infused into the CSF (Table 2). To fix the cannula in position, two stainless steel screws were implanted into the bone and thereafter dental acrylate was poured over the bone hole, screws, external end of the cannula and the connection of the cannula to the plastic tubing, while the osmotic mini-pump was positioned subcutaneously. Finally, the wound was sprayed with local anaesthetic (lidocaine),

and the cat received 50 mL of 5% dextrose intraperitoneally. The next day the cat recovered, moved freely and began to eat spontaneously. Seven days after the operation, the animals were anaesthetized and the CSF sample (1.0 mL) from CM was taken and ICP was recorded as well. Then the animals were sacrified by an anaesthetic overdose, a few drops of trypan blue were given through infusion cannula, and the cannula position in situ was verified by brain dissection, which was previously perfused through the carotid arteries with 200 ml of saline followed by 500 ml 10% formalin at a pressure of 100 cm H_2O (Miše *et al.,* 1996). The size of the brain ventricles was measured by the previously described planimetry (Miše *et al.,* 1996). Three coronal slices, 0.5 mm thick, were taken at the labelled coordinates level (4.5; 9.5 and 14.5 mm anterior from the zero level of stereotaxic frame) for planimetry. The slices and millimetre scale were projected onto a screen at a distance of 50 cm. The contours of the brain slice and ventricle were copied onto millimetre paper and their surface measured by planimetry (see Figs. 5b and 6b). The ventricular surface percentage (%) was obtained by dividing the ventricular surface with the total surface of the slice and multiplying it by 100.

All the results are shown as the mean and standard error of mean (SEM) with the number of experiments/animals (n). For statistical evaluation, a Student's t-test was used and $p<0.05$ was taken as statistically significant.

RESULTS

The appearance/transition of water (^3H_2O) from the blood into iso-osmolar and hyperosmolar CSF was studied by means of the micro-volume exchange method in dogs (Klarica *et al.,* 1994; Fig. 2a; see Methods). CSF osmolarity was increased in the RLV through the application of hyperosmolar sucrose solution (950 mmol/L). In Figure 3, the appearance/transition of ^3H_2O from the blood into the CSF system and the effect on ICP is shown. It can be seen (Fig. 3a) that after the application of ^3H_2O into the blood (femoral vein), the concentration of ^3H_2O in the arterial blood (femoral artery) continuously decreased over 35 minutes. The ^3H_2O influx into the hyperosmolar CSF part (RLV) is significantly higher and faster than into the iso-osmolar CSF (LLV and CM). For example, the ^3H_2O equilibrium between the blood and hyperosmolar CSF was achieved at approximately 8^{th} minute, but between the blood and iso-osmolar CSF at approximately 20^{th} minute (LLV and CM). In addition, the concentration of ^3H_2O in the 8^{th} minute was two times higher in the hyperosmolar CSF part (RLV) than in the iso-osmolar CSF (LLV and CM). The influx of ^3H_2O into the LLV and CM is a consequence of the free exchange of water between the blood and CSF (Bulat *et al.,* 2008), and that the higher and faster influx of water from the bloodstream into the RLV is a consequence of additional osmotic arrival.

In Figure 3b, the effect on ICP in the RLV and CM is shown in the same experimental model. It can be seen that 10 minutes after the sucrose applications into the RLV, ICP increased 200-300% above the control values both in the RLV and CM. The significant increase in ICP lasts more than 80 minutes and in any observed/measured period, there are no statistical differences between ICP in the RLV and CM.

In Figure 4, changes in CSF osmolarity (Table 1; see Methods) and ICP in the RLV are compared over time. When comparing changes in CSF osmolarity and ICP, a significant

increase in RLV CSF osmolarity after the application of hyperosmolar sucrose (950 mmol/L) is noted within the first 30 minutes. It is interesting to note that 50 minutes after the application of hyperosmolar sucrose, normalization in CSF osmolarity was observed whereas ICP still remained significantly high and remained so during the whole time (90 minutes). All of the latter indicates that hyperosmolar CSF osmotically draws water from the bloodstream (Fig. 3a), therefore becoming iso-osmolar, which leads to an increase in CSF volume. Such an increase in CSF volume is reflected in higher/increased ICP, even though the CSF itself has become iso-osmolar in the meantime. The observed phenomenon, which we named "compensated hyperosmolarity", could significantly influence certain pathological conditions of the brain (see Discussion).

Since it was demonstrated in acute experiments that increasing the CSF osmolarity leads to drawing water from the blood into the CSF system and increased ICP, we examined whether the subchronic application of hyperosmolar solution in the RLV would lead to expansion of the ventricles and the development of hydrocephalus. Figure 5 shows the calculated values (5a), the contours (5b) and native form (5c) of the ventricle surface in cats after 7 days of continuous slow infusion of hyperosmolar or iso-osmolar solutions by means of mini-osmotic pumps into the RLV. It can be seen that after the infusion of hyperosmolar sucrose, the ventricular surface is significantly larger (5 a,b,c) in comparison to the control iso-osmolar infusion (5 a,b,c) in any of the observed slices. In Figure 6, the calculated values (6a), the contours (6b) and native form (6c) of the LLV and RLV in cats was shown after 7 days of continuous infusion of hyperosmolar sucrose by means of mini-osmotic pumps into the RLV. The enlargement was significantly greater in the ventricle which was continuously osmotically loaded (RLV) than in the contralateral ventricle (LLV).

In Table 2, ICP and CSF osmolarity in the CM in cats is shown after seven days of iso-osmolar or hyperosmolar solution infusion by means of mini-osmotic pumps into the RLV. It

can be seen that after the infusion of hyperosmolar sucrose, CSF osmolarity in the CM slightly increased, but ICP remained the same as after an iso-osmolar infusion.

These experiments clearly indicate that the loading of the CSF osmolarity caused by a slow infusion of hyperosmolar solution for 7 days resulted in a significant ventricular enlargement and the development of hydrocephalus with a completely open CSF system and without significant increase in ICP (see Discussion).

DISCUSSION

It is very important to stress that 99% of CSF is water, and that when studying CSF hydrodynamics it is crucial to study the hydrodynamics of water. In addition, according to our hypothesis (Orešković and Klarica, 2010; Bulat and Klarica, 2011; Orešković and Klarica, 2011), CSF is not produced actively and cannot be passively absorbed into the venous sinuses, but freely exchange within the entire CSF system (Fig. 1b). The hydrodynamic exchange network of widespread blood capillaries within the CNS (Orešković and Klarica, 2010; Bulat and Klarica, 2011; Orešković and Klarica 2011) is ultimately responsible for this. Due to the large surface area of blood capillaries and the amount of blood flowing through them, it is of crucial importance to observe/study how water from the bloodstream behaves compared to CSF. This is especially important in the case of hydrocephalus, which presents an excessive accumulation of CSF (i.e. water) in the CSF system. It has recently been proposed that development of hydrocephalus (Krishnamurthy *et al.,* 2009; Orešković and Klarica, 2011) and the change in CSF volume is caused by changes in blood or CSF osmolarity (Hochwald *et al.,* 1974; Wald *et al.,* 1976; Orešković *et al.,* 2002; Maraković *et al.,* 2010; Jurjević *et al.,* 2012) and that elevated CSF osmolarity should be considered as one of the most important factors in excessive CSF/water accumulation. We have also presumed that the greatest responsibility for the maintenance of CSF iso-osmolarity is the rapid extraction of water from the bloodstream into the osmotically loaded CSF space, and that extracted water would consequently cause an accumulation of fluid, increase in ICP and the development of hydrocephalus.

Following the behavior of $^{3}H_2O$ in a dog model in which there are both physiological (iso-osmolar CSF; LLV and CM) and pathophysiological conditions (acute hyperosmolar CSF, RLV), we have shown that the entry of water ($^{3}H_2O$) from the blood into the CSF

significantly differs (Fig. 3). The concentration of ^3H_2O in the LLV and CM (iso-osmolar CSF) shows the expected physiological free exchange of water between the blood (cerebral capillaries and CP) and CSF. ^3H_2O concentration within these two CSF compartments did not differ and after 20 minutes was equal to the concentration in the blood. However, in a pathophysiological state (acute hyperosmolar CSF, RLV) ^3H_2O transition from the blood into the hyperosmolar CSF behaved considerably differently. Thus, the ^3H_2O concentration between the blood and CSF was already equal by the eighth minute, and in all measured intervals before equalization there was a significantly higher concentration of ^3H_2O in relation to iso-osmolar CSF (LLV, CM). This entry of ^3H_2O into the CSF was so fast that it was already statistically significant after one minute (Fig. 3).

These obtained results suggest two very important facts. In the field of pathophysiology, acutely osmotically loaded CSF affects the rapid entry of water from the blood circulation into CSF and its potential accumulation as well as the dilatation of the ventricle if CSF osmolarity increase takes a long time. In the field of physiology, overlapping ^3H_2O curves within the LLV and CM suggest that the mechanism of water entering from the bloodstream into these compartments is the same in terms of dynamics and volume. However, in accordance with the "classical" hypothesis, these two curves should differ substantially. Namely, if CSF formation is an active process and takes place in the CPes, then the amount of ^3H_2O in the LLV should be expected to be considerably higher than that obtained by the free exchange of fluid in the CM. These results are in accordance to our previous studies where we have shown that net CSF formation exclusively by CP does not exist (Orešković *et al.,* 2001; Orešković *et al.,* 2002; Bulat *et al.,* 2008; Klarica *et al.,* 2009; Orešković and Klarica 2010; Orešković and Klarica, 2011).

In addition, although the research of Davson *et al.,* (1970; 1973; 1987) did not associate CSF osmolarity with the pathophysiology of ICP and despite the fact that a pathological process

that would lead simultaneously to an increase in CSF osmolarity and ICP in the CNS is not known in the literature, we assumed, based on our preliminary results (Klarica *et al.,* 1998) and the described effect of hyperosmolar CSF that ICP should be elevated. Therefore, we have measured ICP using the same model after the acute application of hyperosmolar sucrose into the RLV (Fig. 3b). It was clearly demonstrated that an acute increase in CSF osmolarity caused an increase in ICP. The entry of water caused by the osmotic force (Fig. 3a) is so important for the ICP increase, that the application of hyperosmolar sucrose led to a doubling of ICP throughout the CSF system (RLV, CM) within 4 minutes, with ICP remaining elevated during the entire measurement (Fig. 3b). Since sucrose cannot considerably penetrate cell membranes, it is not possible to ascribe such a large and fast increase in ICP to the diffusion of sucrose into the brain parenchyma and the possible development of a periventricular brain edema. This was also supported by the absence of any significant changes in periventricular density verified in this model on a CT scan (Klarica *et al.,* 1994). Thus, it is obvious that the increase in ICP is related to acute osmotically loaded CSF and the consequent accumulation of water in the CSF system.

We also believe that the perceived decline of CSF osmolarity in the RLV (Table 1) primarily happens due to the rapid osmotic entry of water from the bloodstream into the CSF (Fig. 3a). We expected that sucrose molecules being slowly transferred by CSF pulsations into the ISF (as other substances with similar m.w. and chemical characteristics), other CSF compartments (Bulat and Klarica, 2011) and blood circulation contributes to a lesser extent to the normalization of CSF osmolarity, since larger molecules are also partially absorbed into the blood (Davson *et al.,* 1989). However, comparing the normalization of CSF osmolarity and ICP at the same intervals, we found that CSF became iso-osmolar, but ICP still remained significantly elevated (Fig. 4). This phenomenon of elevated ICP (caused by osmotically loaded CSF) after CSF hyperosmolarity had disappeared, was named “compensated

hyperosmolarity". Perhaps the existence of "compensated hyperosmolarity" could explain why until now an increase in CSF osmolarity in patients with elevated ICP has not been observed. Namely, if the pathological process occurs in a patient as a result of increased CSF osmolarity, it would cause a rapid osmotic entry of water into the CSF system, on the one hand leading to an increase in ICP, and to the rapid onset of iso-osmolarity on the other. "Compensated hyperosmolarity" is also difficult to detect, because even if hyperosmolarity occurs within an isolated CSF compartment (only within the RLV; Table 1), it would still lead to a pathological response (elevated ICP) within the entire CSF system (within the ventricles and SAS; Fig. 4). It would be especially difficult to see increased CSF osmolarity if the place from where the CSF is taken (usually the lumbar SAS) is far from the place where the pathological process takes place, since we have shown that despite the application of hyperosmolar sucrose into the RLV, CSF remained iso-osmolar in the LLV and CM (Table 1). Besides elevated ICP, a similar consideration would be true in the case of hydrocephalus. For the reasons mentioned, it is impossible to see either an increase in CSF osmolality at higher ICP or an increase in CSF osmolality in patients with hydrocephalus, because the accumulation of CSF and ventricular enlargement occur due to the osmotic entry of water until CSF iso-osmolarity is attained. "Compensated hyperosmolarity" is therefore a very important observation, because when iso-osmolar CSF is obtained in biochemical analysis, it could often be wrongly concluded that osmotic loading of CSF did not cause increased ICP or the development of hydrocephalus. We assume that various pathological processes in the brain (infection, ischemia, trauma, inflammation, bleeding, etc.) may increase CSF osmolarity due to either damage of the brain parenchyma and increased diffusion of certain substances into the CSF or due to the impaired transport of certain substances from the CSF into the bloodstream, hence causing these substances to accumulate in the CSF and resulting in an increase in CSF osmolarity. Thus, if CSF hyperosmolarity is not noted, it should always be

kept in mind that this does not necessarily exclude osmotic loading of CSF as the cause of the development of high ICP or hydrocephalus or some other pathological process.

In addition, if CSF in dogs circulates at 30-50 μL/min from the ventricles to the CM (as is suggested by the "classical" hypothesis), it should not be expected that in 35 minutes (life time of hyperosmolarity in RLV) the hyperosmolar sucrose carried by CSF circulation from the ventricle to the CM would not lead to at least a tendency for an increase in CSF osmolarity in CM, which is situated only ten centimeters away from the RLV. Therefore (under such circumstances), CSF iso-osmolarity in the CM also indicates that unidirectional CSF circulation does not exist and that the substances in the CSF systems are not being carried away by CSF circulation from the ventricles to the site of absorption (arachnoid villi). According to our hypothesis (Orešković and Klarica, 2010; Bulat and Klarica, 2011; Orešković and Klarica 2011), the distribution of substances in the CSF system is performed by CSF pulsations in all directions, and the distributed distance depends on the half-life of the substances in the CSF.

Using sub-chronically infused hyperosmolar solution via an osmotic mini-pump into the RLV CSF in cats, hydrocephalus was caused (Figs. 5 and 6). We can say that it is obvious that a long-term (7 days) increase in CSF osmolarity leads to the development of hydrocephalus, as was observed in rats (Krishnamurthy *et al.,* 2009). However, while increased osmolarity in rats led to an equal development of hydrocephalus in the whole ventricular system, in our experiments hydrocephalus predominantly developed in the RLV (Figs. 5 and 6). The reason could be the two times longer duration of the hyperosmolar infusion in rats, which could have led to the osmotic entry of water into the infused ventricle and the consequent spreading of the volume through the entire ventricular system. It is important to note that in rats (Krishnamurthy *et al.,* 2009) as well as in cats, none of the animals with hydrocephalus had obstruction of CSF pathways and that ICP was not affected (Table 2). In other words, if the

application of hyperosmolarity is slow and time-consuming, it probably results in a slow entry of water from the blood and does not lead to elevated ICP. In experiments on dogs, where the hyperosmolarity application was acute, the entry of water from the blood was very rapid and led to a multiple and sharp rise in ICP (Fig. 4).

Thus, the existing total volume of CSF is not the result of the CSF volume secreted from the CPes and CSF volume absorbed into the venous sinuses, but the result of CSF/water exchanges within the entire CSF system with fluid from surrounding tissue and the bloodstream. Therefore, it is necessary to point out that a change in CSF osmolarity does not affect CSF formation/secretion (Orešković *et al.,* 2002), as was believed during earlier investigations (Hochwald *et al.,* 1974; Wald *et al.,* 1976). Just as it was clearly demonstrated in our previous publications that net CSF formation does not exist (Orešković *et al.,* 2002; Klarica *et al.,* 2009; Maraković *et al.,* 2010; Orešković and Klarica, 2010), so in this work on dogs by following the water from the bloodstream into the CSF system in iso-osmolar and osmotically loaded CSF (Fig. 2), we have clearly shown that the entry of water/CSF in any case does not happen by means of so-called CSF "secretion", but is based on hydrodynamic laws. Osmotically loaded CSF leads to a greater entry of fluid volume into the CSF system (from the bloodstream and surrounding tissue) that in our acute application experiments resulted in elevated ICP (Figs. 4 and 5) or, in the case of sub-chronic application, in the development of hydrocephalus (Figs. 5 and 6).

CONCLUSION

For the first time experimentally, an increased entry of water from the blood (cerebral capillaries and CP) into only the hyperosmolar part of the CSF with a simultaneous physiological free exchange of water in the rest of the CSF iso-osmolar system was demonstrated. For the first time, the relationship between osmotically loaded CSF and an elevated ICP was clearly shown. For the first time, the existence of "compensated hyperosmolarity" in a model developed in our laboratory was shown and explained. It was confirmed that sub-chronic constant application of hyperosmolar solution in cats resulted in hydrocephalus development without any obstruction of CSF pathways. It was shown that a local and continuous increase in CSF osmolarity leads to a greater spread of CSF spaces at the site of application, which explains the occurrence of unilateral hydrocephalus without the blocking of CSF pathways. All this strongly supports our idea that CSF hyperosmolarity could be a factor in hydrocephalus etiology. Finally, these presented results clearly confirm the hypothesis of a new CSF hydrodynamics and a new hypothesis about the hydrocephalus pathophysiology (Orešković and Klarica, 2010; 2011) and certainly cannot fit into the classical hypothesis of secretion, circulation and absorption of CSF. We, therefore, hope that this new approach to CSF hydrodynamics can help to understand CSF-related diseases and develop new strategies for treatment.

Acknowledgements

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Figure Legends

Figure 1. Schemes of cerebrospinal fluid hydrodynamics. The central nervous system is shown as a grey area and cerebrospinal fluid as white. **a)** The scheme represents the classical hypothesis of cerebrospinal fluid secretion, unidirectional circulation and absorption. The arrows point in the direction of cerebrospinal fluid circulation and the sites of cerebrospinal fluid absorption. **b)** The scheme represents a new hypothesis of cerebrospinal fluid hydrodynamics as an interrelation between cerebrospinal fluid, interstitial fluid and cerebral blood vessels. The arrows represent the widespread exchange of water and substances between the blood and interstitial-cerebrospinal fluid through the blood-brain barrier.

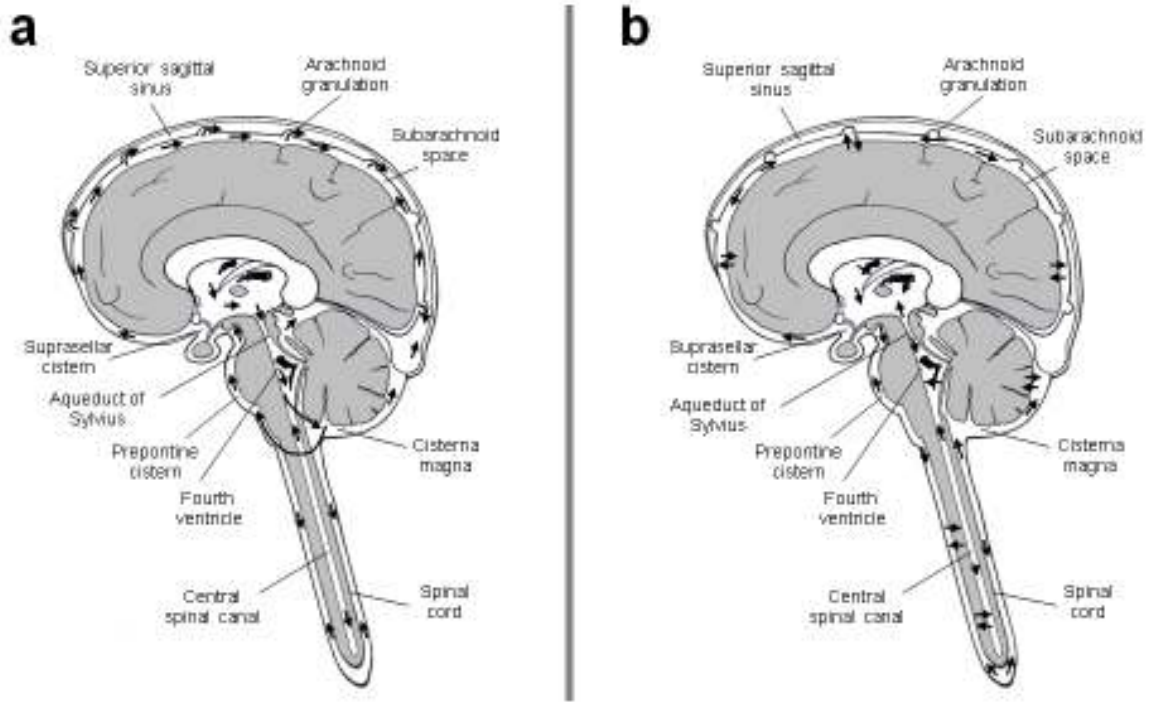


Figure 2. a. Scheme of the micro-volume exchange method in dogs. **b.** The effect of the application of different hyperosmolar sucrose solution (mmol/L) in the right lateral ventricle in dogs on intracranial pressure (cm H_2O) by means of the micro-volume exchange method. The arrow shows the time of sucrose application. A significant increase in intracranial pressure was calculated against the control intracranial pressure (0 time), and "n" represents the number of animals.

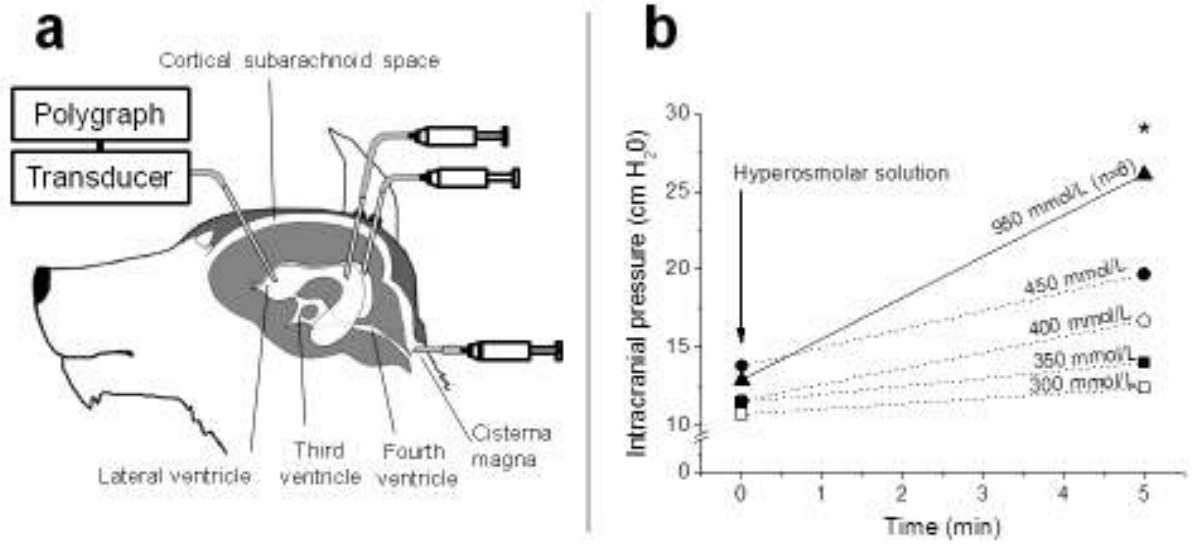
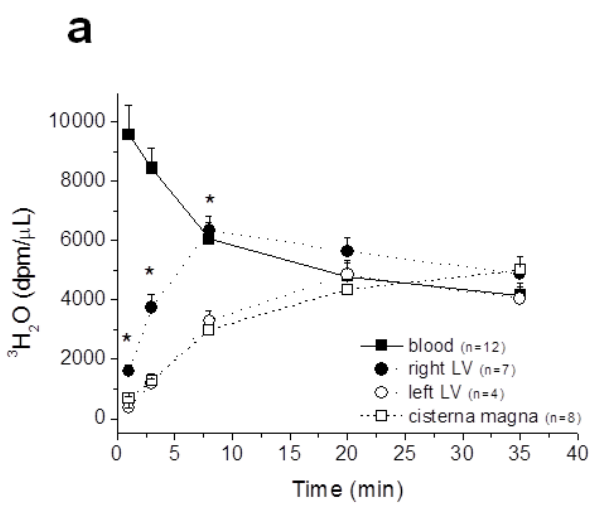


Figure 3. The results obtained by means of the micro-volume exchange method in dogs. **a.** The change in $^{3}H_2O$ concentration (dpm/50 μL) over 35 minutes in arterial blood (femoral artery) in iso-osmolar (cisterna magna and left lateral ventricle) and hyperosmolar (right lateral ventricle) cerebrospinal fluid after the application of 4 mL $^{3}H_2O$ (time "0"; $9x10^{6}$ dpm/50 μL) into the femoral artery. The results are shown as a mean value ± SEM, and "n" represents the number of experiments. $^{*}p<0.001$ **b.** The effect of hyperosmolar sucrose solution (950 mmol/L H_2O; 1 mL) applied into the right lateral ventricle on intracranial pressure measured in the lateral ventricle and cisterna magna. The arrow shows the time of sucrose application. The horizontal broken line represents the projection of the control values of intracranial pressure. The significant difference in intracranial pressure was calculated against the control intracranial pressure before application, and "n" represents the number of animals. The results are shown as a mean value ± SEM. $^{*}p<0.001$; $^{+}p<0.002$; $^{x}p<0.01$; $^{y}p<0.05$.


a
10000
8000
6000
4000
2000
0
^3H_2O (dpm/μL)
0
5
10
15
20
25
30
35
40
Time (min)
blood (n=12)
right LV (n=7)
left LV (n=4)
cisterna magna (n=8)

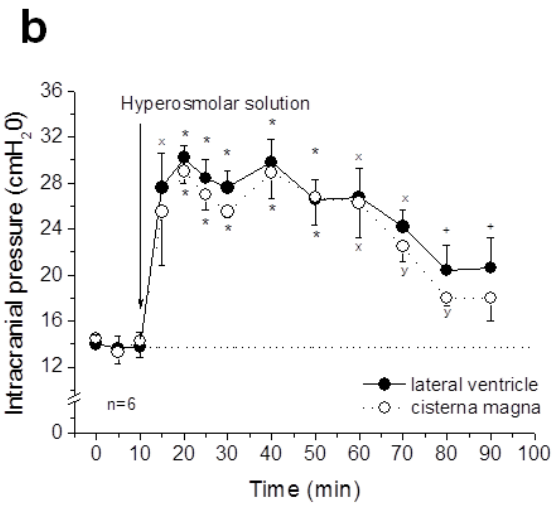

b
Hyperosmolar solution
36
32
28
24
20
16
12
0
Intracranial pressure (cm$H_2$0)
n=6
0
10
20
30
40
50
60
70
80
90
100
Time (min)
lateral ventricle
cisterna magna

Figure 4.

Changing the CSF osmolarity (mmol/kg H_2O; vertical bars) and intracranial pressure (cm H_2O; black squares) measured in the right lateral ventricle after hyperosmolar sucrose application (950 mmol/L H_2O; 1 mL) into the right lateral ventricle by means of the micro-volume exchange method in dogs. The arrow shows the time of sucrose application. The horizontal broken lines represent the projections of the control values of intracranial pressure (cm H_2O) and cerebrospinal fluid osmolarity (mmol/L H_2O). The numbers in the vertical bars and "n" represent the number of animals. The results are shown as a mean value ± SEM. The significant difference in intracranial pressure and cerebrospinal fluid osmolarity was calculated against the control intracranial pressure and cerebrospinal fluid osmolarity before application. $^{*}p<0.001$; $^{+}p<0.002$; $^{x}p<0.01$; $^{y}p<0.05$

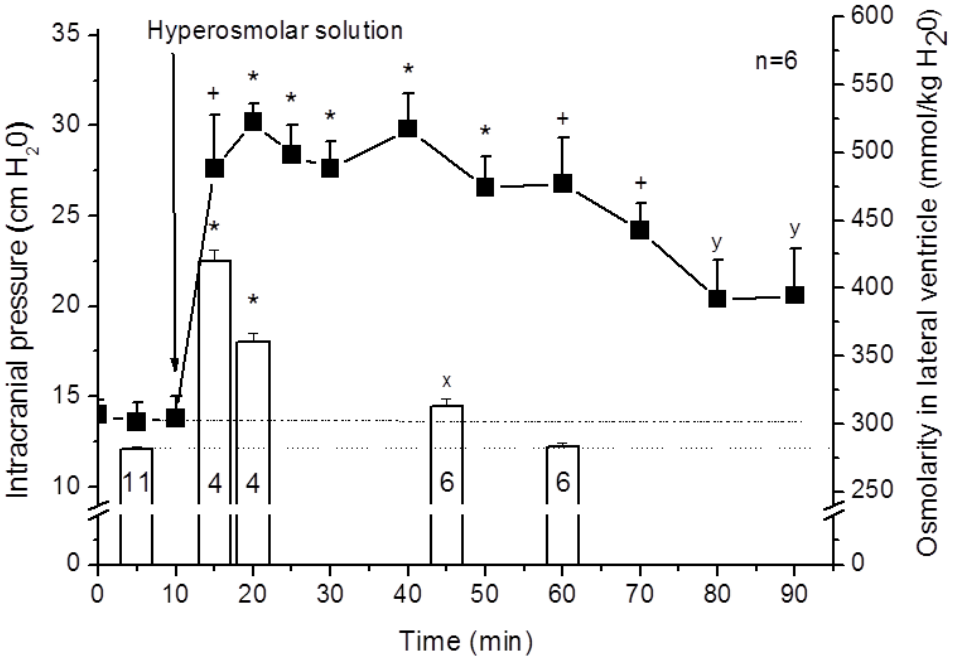

Hyperosmolar solution
n=6
Intracranial pressure (cm H_2O)
Osmolarity in lateral ventricle (mmol/kg H_2O)
Time (min)
35
30
25
20
15
10
0
600
550
500
450
400
350
300
250
0
0
10
20
30
40
50
60
70
80
90
11
4
4
6
6
x
y
y
+
*

Figure 5.

a. Surface of cat brain ventricles (%) at different stereotaxic coordinates (A4.5; A9.5; A14.5 mm) after seven days of slow infusion of iso-osmolar solution (control; empty bars) or hyperosmolar solution (2400 mmol/L H_2O; grey bars) by means of osmotic mini-pump (rate 10 µL/h) into the right lateral ventricle. The results are shown as a mean value ± SEM. The numbers in vertical bars represent the number of animals. $^{*}p<0.05$ **b.** The contours of brain slices and ventricles at different stereotaxic coordinates (A4.5; A9.5; A14.5 mm) after seven days of slow infusion (10 µL/h) of iso-osmolar (control) or hyperosmolar solution by means of osmotic mini-pump. **c.** Photographs of cat brain slices at different stereotaxic coordinates (A9.5; A14.5 mm) after seven days of slow infusion (10 µL/h) of iso-osmolar (control) or hyperosmolar solution (2400 mmol/L H_2O) by means of osmotic mini-pump.

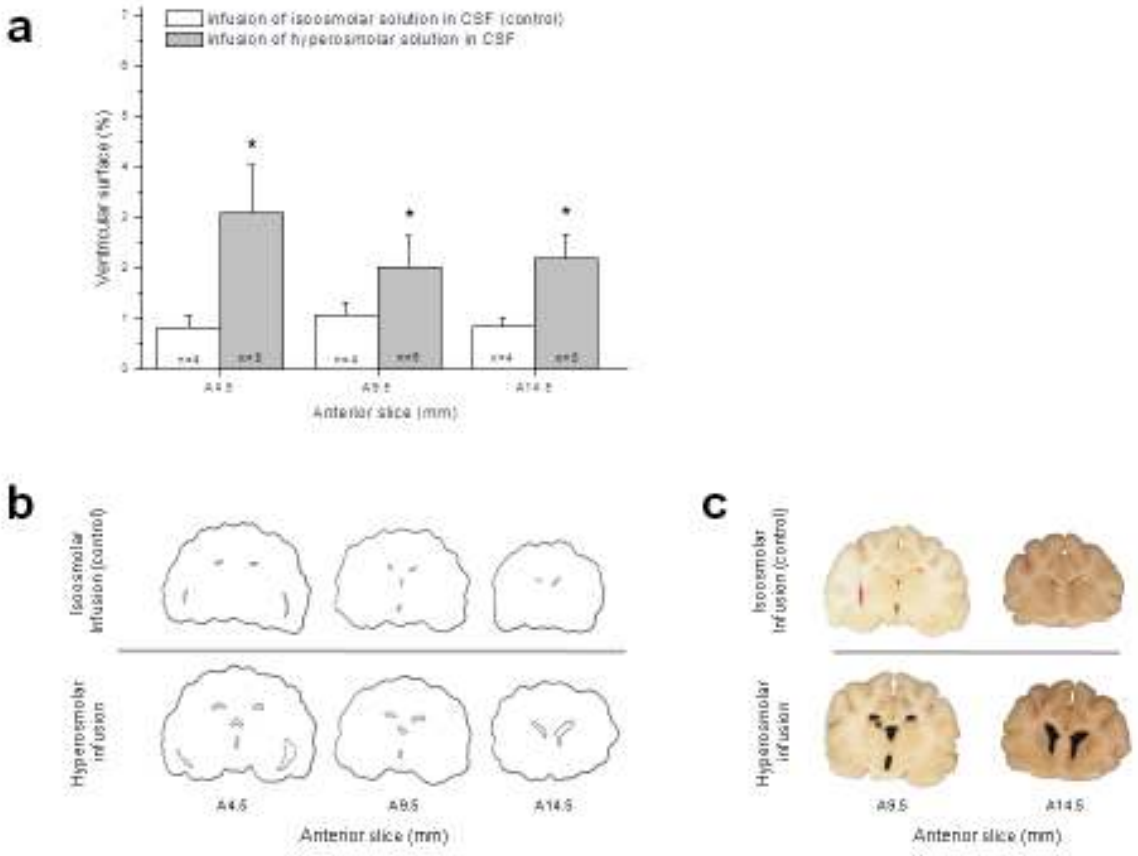

a
Infusion of isoosmolar solution in CSF (control)
Infusion of hyperosmolar solution in CSF
Ventricular surface (%)
Anterior slice (mm)
b
Isoosmolar infusion (control)
Hyperosmolar infusion
A4.5
A9.5
A14.5
Anterior slice (mm)
c
Isoosmolar infusion (control)
Hyperosmolar infusion
A9.5
A14.5
Anterior slice (mm)

Figure 6.

a. Surface (%) of right (RTV; empty bars) and left (LLV; grey bars) brain ventricles in cats at different stereotaxic coordinates (A4.5; A9.5; A14.5 mm) after seven days of slow infusion of hyperosmolar solution by means of osmotic mini-pump (rate10 μL/h) into the right lateral ventricle. The results are shown as a mean value ± SEM. The numbers in the vertical bars represent the number of animals. $^{*}p<0.05$**.** The contour (**b**) and photograph (**c**) of the cat brain surface at A4.5 mm stereotaxic co-ordinates after seven days of slow infusion (10 μL/h) of hyperosmolar solution (2400 mmol/L H_2O) by means of osmotic mini-pump.

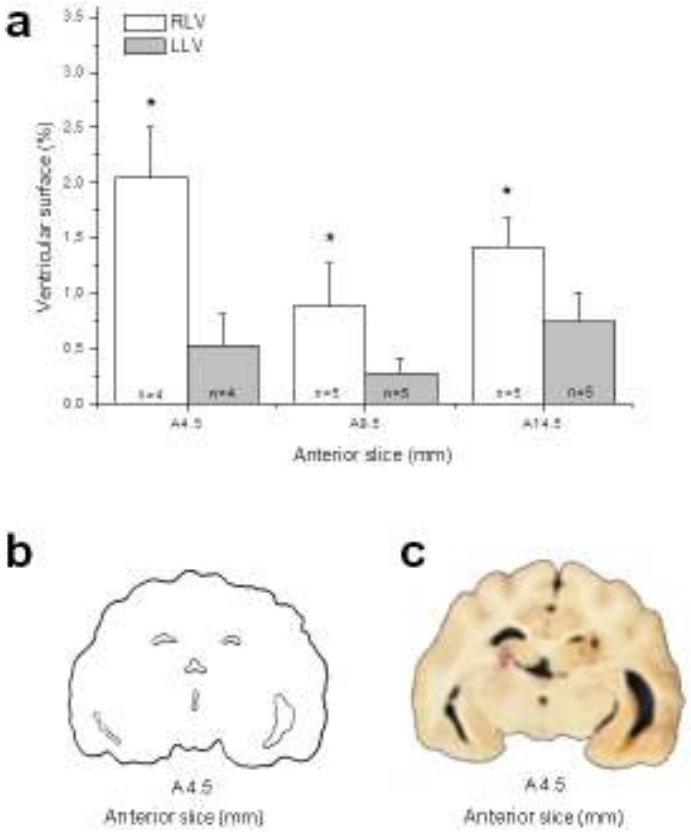


Table 1. Testing hyperosmolar sucrose distribution within the CSF system by microvolume exchange method in dogs

CSF compartment		Cerebrospinal fluid osmolarity (mmol/L H_2O)				
	Time (min)	0	5	10	35	50
LLV (n)			285±4 (3)	280±0 (2)	283±3 (2)	280±0 (2)
RLV (n)		283±6.9 (11)	420±8 (4)*	360±7 (4)*	313±5 (6)**	283±3 (6)
CM (n)		285±4.8 (7)	284±1 (4)	285±0 (3)	280±0 (2)	283±3 (2)

*$p<0.001$ in relation to 0 time
**$p<0.01$ in relation to 0 time

Cerebrospinal fluid osmolarity (mmol/kg H_2O) at different time intervals in the left lateral ventricle (LLV), right lateral ventricle (RLV) and cisterna magna (CM) after application of hyperosmolar sucrose into the right lateral ventricle by means of the micro-volume exchange method in dogs. The significant difference of cerebrospinal fluid osmolarity in the right lateral ventricle was calculated against the cerebrospinal fluid osmolarity in the left lateral ventricle and cisterna magna. The number of experimental animals is shown in brackets. The results are shown as a mean value ± SEM.

Table 2. Intracranial pressure (ICP; cm H_2O) and CSF osmolarity (mmol/L H_2O) in cats cisterna magna (CM) after 7 days ventricular isoosmolar or hyperosmolar infusion by miniosmotic pumps

Infusion of isoosmolar solution into right lateral ventricle			**Infusion of hyperosmolar solution into right lateral ventricle**		
Cat N^o	ICP pressure in CM in cm H_2O	CSF osmolarity in CM in mmol/L	Cat N^o	ICP pressure in CM in cm H_2O	CSF osmolarity in CM in mmol/L
1	6.0	303	5	6.0	311
2	4.0	307	6	7.0	320
3	4.0	303	7	6.0	318
4	7.0	304	8	5.0	315
Mean±SEM	4.7±0.47	304.3±0.83		6.0±0.35	316±1.70*

*$p<0.01$

Intracranial pressure (cm H_2O) and cerebrospinal osmolarity (mmol/kg H_2O) in the cisterna magna (CM) in cats after sub-chronic (7 days) slow infusion (10.0 µL/h) of iso-osmolar or hyperosmolar (2400 mmol/L H_2O) sucrose by means of osmotic mini-pump. The significant difference in intracranial pressure and cerebrospinal fluid osmolarity was calculated between animals with iso-osmolar and hyperosmolar infusion. The results are shown as a mean value ± SEM.

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