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Running head: Brain and muscle water in acute hyponatremia

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Abstract:

Brain edema is suggested to be the principal mechanism underlying the symptoms in acute hyponatremia. Identification of the mechanisms responsible for global and regional cerebral water homeostasis during hyponatremia is therefore of utmost importance. To examine the osmotic behavior of different brain regions and muscles, *in vivo* determined water content (WC) was related to plasma $[\text{Na}^+]$ and brain/muscle electrolyte content. Acute hyponatremia was induced with desmopressin acetate and infusion of a 2.5% glucose solution in anesthetized pigs. WC in different brain regions and skeletal muscle was estimated *in vivo* from magnetic resonance imaging (MRI) determined T$_1$ maps. WC, expressed in gram water per 100 gram dry weight, increased significantly in slices of the whole brain (342(SD=14) to 363(SD=21)) (6%), thalamus (277(SD=13) to 311(SD=24)) (12%), and white matter (219(SD=7) to 225(SD=5)) (3%). However, the WC increase in the whole brain and white matter WC was less than expected from perfect osmotic behavior, whereas in the thalamus, the water increase was as expected. Brain sodium content was significantly reduced. Muscle WC changed passively with plasma sodium concentration. WC determined with deuterium dilution and tissue lyophilization correlated well with MRI-determined WC. In conclusion, acute hyponatremia induces brain and muscle edema. In the brain as a whole and in thalamus RVD is unlikely to occur. However, RVD may in part explain the observed lower WC in white matter. This may play a potential role in osmotic demyelination.

**Keywords:** Animal model, desmopressin, regulatory volume decrease, brain edema, water intoxication
Introduction:

Hyponatremia is the most common electrolyte disorder in clinical practice with a prevalence reaching 30% in hospitalized patients (57). It may be associated with severe cerebral dysfunction and high mortality (6). Conversely, its correction may also cause severe morbidity and mortality (53; 63). Despite the common and severe nature of hyponatremia, the serious neurological complications associated with hyponatremia and its correction are incompletely understood (63), and may therefore not lend themselves easily to rational treatment.

The most important symptoms in acute hyponatremia are cerebral and are believed to stem from cerebral edema. This corresponds well with the observed cellular water influx when the extracellular osmolality drops (5; 7; 22; 29; 34; 35). Several authors have shown, however, that the increase of water in the entire rodent brain is less than should be expected given the decreased extracellular osmolality (7; 22; 29; 54). Initially (hours), this is consistent with a reduction in total brain Na⁺ following a reduction of the cerebral extracellular volume (ECV) (30; 31). Later (hours, days), intracellular potassium and organic osmolytes have been observed to be reduced in rat brains (55) (22; 64). In patients suffering from chronic hyponatremia (more than 2 days), brain organic osmolytes also decreased (44; 66). To our knowledge, no studies have explored cerebral water content (WC) during acute hyponatremia in man; most studies have used rodents whose physiology may differ from that of man. Further, acute hyponatremia in rodents has primarily been induced instantaneously by means of large quantities of water, which have been infused into the peritoneum or the stomach, which may possibly have impaired cardiovascular dynamics and respiratory functions. However, exceptions exist where acute hyponatremia is induced in rats over hours by administration of electrolyte-free water by gavage (55).
A regulatory volume decrease (RVD), as observed in cell cultures, has been quoted as the reason for the imperfection of the brain to act as an osmometer in acute hyponatremia. However, when cells \textit{in vitro} are exposed to hypotonic media, they immediately (seconds) expel potassium and chloride and later (minutes/hours) extrude organic osmolytes to reduce volume (21). The time course of the volume regulation observed \textit{in vitro} thus differs significantly from that in rodents. This may be due to different mechanisms: 1) In the cell studies, tonicity changes are extreme (50% reduction) and abrupt (seconds); 2) isolated cells act differently from cells in an integrated \textit{in vivo} setting; and 3) differences between brain cells have been demonstrated (40). This is consistent with results from a study of brain slices where cortical brain cells showed no evidence of RVD (3). The challenge for research is to study physiological changes at the cellular level \textit{in vivo}. Magnetic resonance imaging (MRI) offers an opportunity for studying an assembly of cells in their original anatomy without disrupting their physiology (16). This makes it possible to study acute hyponatremia, not only \textit{in vivo}, but also in different brain regions simultaneously.

Plasma $[Na^+]$ is determined by total body water, exchangeable sodium and exchangeable potassium (9). It is therefore important to examine the entire system/body when modeling plasma $[Na^+]$ in the treatment or prevention of hyponatremia. For this reason the skeletal muscles are of primary interest as muscles contains 50% of total body water (TBW) and 70% of total body potassium compared with the ECV that contains only 35% of TBW and only 1-2% of total body potassium (58). Furthermore, it was recently demonstrated that plasma $[Na^+]$ can be predicted from external water and electrolyte balances in a pig model of acute hyponatremia. This indicates that acute (hours), massive RVD with efflux of intracellular electrolytes and organic osmolytes was unlikely to occur in skeletal muscle (38). This is also consistent with studies of single muscle cells (10; 19; 39) and an \textit{in vivo} study of rat diaphragm (8).
which showed that muscle cells act as perfect osmometers within a time span of a few hours. On the other hand, studies have demonstrated RVD or indicated the presence of RVD in connection with changes in the transport of organic osmolytes in muscle cells and myotubes derived from skeletal muscle (17; 26; 37). Others have shown a lesser WC in isolated rat muscle than should be predicted from the change in osmolality (4). Consequently, the existence of acute RVD in muscle cells remains controversial (48).

The aim of the present study was therefore to examine brain and skeletal muscle WC in relation to plasma [Na+] \textit{in vivo}. The WC was measured simultaneously in different brain regions and muscles using serial MRI in a previously described clinically relevant model of acute hyponatremia in pigs (38).

Materials and methods

\textit{Animal preparation.}

Fifteen female Landrace/Yorkshire crossbreed pigs (28-37 kg) were studied. The study complies with national and international regulations for animal research and welfare. The experiment has been approved by the National Animal Ethics Committee (Dyreforsøgstilsynet, Copenhagen, Denmark: License 2007-561-1399).

The pigs were fasted overnight with free access to water. Before transport to the research facility, the animals were sedated with midazolam (Dormicum®, Hameln Pharmaceuticals GmbH, Hameln, Germany) 0.5 mg/kg and azaperone (Stresnil®, Janssen-Cilag GmbH, Neuss, Germany) 1 mg/kg i.m. Anesthesia was induced with etomidate (Hypnomidate®, Janssen Pharmaceutica N.V., Beerse,
Belgium) 0.5 mg/kg intravenously and maintained with a continuous infusion of S-ketamine (S-ketamine®, Pfizer ApS, Ballerup, Denmark) (5-14mg/kg/hour) and midazolam (1-3mg/kg/hour). The animals were intubated endotracheally (6.5mm Portex Tracheal Tube, Smiths Medical International Ltd.), and mechanically ventilated (Servo 900D, Siemens-Elema, Stockholm, Sweden) using volume-controlled positive-pressure ventilation (positive end expiratory pressure: 5 cmH2O; tidal volume: 8-10 mL/kg; respiratory frequency (RF): 15-20 min⁻¹; inspiratory/expiratory-ratio: 1:1; Fraction of inspiratory O2 was 0.5). Ventilation was adjusted to keep end tidal CO2 between 5.5 and 6.0 kPa (20).

Surgical procedures.

Before surgical procedures, the animal received 750 mg i.v. cefuroxim Stragen (Stragen Nordic, Stenlose, Denmark) and the skin was prepared with a 0.5% chlorhexidine/85% ethanol solution. A Radifocus® Introducer II Fr.8, (Terumo Corporation, Tokyo, Japan) was inserted by an open approach into the external jugular vein (for fluids and drug administration). A Radifocus® Introducer II, Fr.6 (Terumo Corporation, Tokyo, Japan) was inserted into the common carotid artery (for measuring blood pressure and heart rate and for blood sampling). A supra pubic bladder catheter was inserted by an open procedure for precise urine collection (20 Ch Foley Catheter (Unomedical, Kedah, Malaysia)). Skin wounds were meticulously closed with 2-0 Ethilon (Johnson and Johnson Intl, Hamburg, Germany).

Experimental protocol.

Animals were randomly allocated to one of two groups. After the first MRI scanning, one group (n=8) received a single intravenous dose of 4 μg desmopressin acetate (DDAVP) (Minirin®, Ferring AB, Limhamn, Sweden) and a 2.5% glucose solution (Glucose 50 mg/ml, Fresenius Kabi AB, Uppsala, Sweden mixed 1:1 with sterile water, Baxter S.A., Lessines, Belgium) was infused at a rate of 10
ml/kg/hour together with Ringer Lactate (Na⁺: 130 mmol/l, K⁺: 4 mmol/l, Ca²⁺: 1,5 mmol/l, Cl⁻: 109 mmol/l, Lactate: 28 mmol/l, Osmolality: 260 mOsm/kg H2O, Fresenius Kabi AB, Uppsala, Sweden) 5 ml/kg/hour for seven hours (the model has been described in detail elsewhere (38)). The other group (n=7) did not receive desmopressin and was infused with Ringer Lactate at a rate of 5 ml/kg/hour for eight hours. Blood pressure and heart rate were monitored continuously (CardioMed 4008, Oslo, Norway). The body weight prior to and after the experiment was measured (Soehnle Professional GmbH & Co. KG, Backnang, Germany). Muscle biopsies (100-200 mg) were taken from the masseter muscle at time zero and from the contralateral muscle at time 420 min and the biopsies were immediately weighed (Mettler AT201 DeltageRange®, Mettler-Toledo A/S, Glostrup, Denmark) and frozen at minus 80° Celsius. After euthanasia (3 ml/kg Pentobarbital 200 mg/ml, The Veterinary Pharmacy, Copenhagen University, Copenhagen, Denmark), the entire brain was removed in seven animals (hyponatreemic: n=3, control n=4) and was immediately frozen at minus 80° Celsius.

Scanning protocol.

MRI was performed on a 1.5 Tesla General Electric Twinspeed Scanner (Milwaukee, WI, USA) using a standard quadrature head coil. The fully anesthetized pig was placed in supine position and not moved further throughout the entire experiment. The applied imaging protocol is shown in Figure 1. Based on a sagittal localizer, 24 T₁-weighted images were recorded: (TR=400 msec., TE = 12 msec. FOV= 30 cm, 24 slices slice thickness= 3 mm, 256x192, Fast Spin Echo and (TR=3000msec,TE=30 msec., FOV = 30 cm, slice thickness=3mm, 384x256, Spin Echo), respectively. The slices were located coronally using the midpoint in the medulla oblongata and the most frontal part of the brain as fix
points. The masseter muscle was included in the slices as well. Identical angulations were used in seven inversion recovery sequences for estimation of tissue T1 values (TE=10 msec. TR=3000 msec., FOV 30 cm, 256x156, eight slices, IR= 200, 400, 600, 800, 1000, 1200, and 1400 msec., respectively). Finally, for estimation of the apparent diffusion coefficient (ADC), diffusion-weighted images (DWI) were recorded (15 slices, FOV=30 cm, TE=85.3 msec. TR= 4000 msec, FOV= 30 cm, slice thickness = 5 mm, 128x128, EPI, b=0 and b = 1000 s/mm²). This full set of recordings was repeated six times over the next seven hours.

*Cerebral and muscle water content (MRI).*

Based on the inversion recovery recordings, tissue T1 was estimated and according to the equation $1/W = A + B/T_1$ ($W$: gram water per gram tissue, $T_1$: value estimated from the inversion recording, $A$ and $B$ are constants), tissue water was estimated for each pixel in the images (16; 60). The constants $A$ and $B$ were determined using tissue-mimicking gelatine (Gelatine for Microbiology, Merck, Darmstadt, Germany) standards of different WC (68 – 95) (16). The calculation of T1 maps was performed in MISTAR (Apollo Imaging Technology, Melbourne, Australia) using a two parameters least squares regression. The T1 maps were manually segmented into four regions of interest: whole brain, thalamus, white matter, and the most homogeneous part of the masseter muscle (27) (Figure 2).

*Relative water diffusion (ADC)*
The MISTAR program (Apollo Imaging Technology, Melbourne, Australia) was used in a pixel wise calculation of the ADC maps: \[ \text{ADC} = \ln\left(\frac{S(0)}{S(b)}\right)/b \], where \( S(b) \) is the signal intensity using a diffusion-weighting factor \( b \), and \( S(0) \) is the signal intensity at \( b=0 \).

**Laboratory measurements.**

Blood samples were drawn before intervention and successively every second hour from the common carotid artery into lithium-heparin tubes and centrifuged for 10 min at 3500 RPM. Plasma was analyzed for [Na\(^+\)], [K\(^+\)], [creatinine], [urea], [albumin] and [Mg\(^{2+}\)] using a Vitros 5.1 FS analyzer (Ortho-Clinical Diagnostics, Copenhagen, Denmark).

Arterial blood was analyzed (ABL system 700, Radiometer, Copenhagen, Denmark) to determine pH, pO\(_2\), pCO\(_2\), [base excess], [Standard-HCO\(_3^-\)], [hemoglobin], hematocrit level, [glucose], [lactate\(^-\)], [Na\(^+\)], [K\(^+\)], [Cl\(^-\)] and [Ca\(^{2+}\)].

Urine was collected every second hour and urine [Na\(^+\)] and [K\(^+\)] were determined on an Eppendorf Flame Photometer (Eppendorf, Hamburg, Germany) and [creatinine], [urea], [albumin], [glucose] and [Mg\(^{2+}\)] on a Vitros 5.1 FS analyzer (Ortho-Clinical Diagnostics, Copenhagen, Denmark).

Plasma and urine for determination of osmolality were immediately frozen at minus 80° Celsius. Osmolality was measured after thawing using an Advanced® Model 3900 Multi-Sample Osmometer (Advanced Instruments, Massachusetts, USA).
Brain and muscle electrolytes.

The frozen brains were transected with a saw. Half of the brain was weighed (Sartorius, Bie and Berntsen A/S, Roedovre, Denmark) and immediately transferred to air- and water-tight bottles (Schott Duran® 500 ml, DURAN Productions GmbH and Co.KG, Mainz, Germany). The brains were extracted using 350 ml 5% (w/v) trichloroacetic acid (Fluka, Buchs, Switzerland) (TCA) for seven days. The solution was filtered (Natgene® 90 mm, 500 ml, Natgene Nunc, N.Y., USA), and Na⁺ and K⁺ content of the TCA extracts were determined with the use of a FLM3 flame photometer (Radiometer, Copenhagen, Denmark) with lithium as the internal standard. WC determined using MRI was used to calculate electrolyte content per gram dry weight in each brain. Muscle biopsies were extracted in 4 ml 5% TCA in air- and water-tight CryoTubeVials® (Nunc A/S, Roskilde, Denmark) for three days and Na⁺ and K⁺ content were determined as in the brain extract. WC determined using lyophilization was used to calculate electrolyte content per gram dry weight in each muscle.

Cerebral water content (deuterium oxide).

The deuterium concentration in the cerebral solution described in the previous paragraph was determined by mass spectrometry (38). By applying the principle of dilution, the water volume in the brain (V_{CNS}) was determined (see appendix for derivation):

\[ V_{cns} = V_{tca} \frac{(D_{tca} - D_{sol})}{(D_{sol} - D_{plasma,420})}, \]

where \( V_{tca} \) = volume TCA added to the brain; \( D_{tca}, D_{sol}, D_{plasma,420} \) are fractional abundances of deuterium in the TCA, the brain solution after seven days and in plasma at time 420 min.
Muscle water content.

Muscle biopsies were weighed (Mettler AT261 DeltaRange®, Mettler-Toledo A/S, Glostrup, Denmark) immediately after removal from the animal and after lyophilization (HETO CT110, LH Laboratorie Service, Hoerning, Denmark). The WC was calculated as the difference between wet and dry weight. For unknown reasons, two late biopsies from hyponatremic animals had very high WCs, resulting in an apparent more than 80% increase in WC between time zero and 420 min (Figure 3). Four biopsies from control animals contained tendon. We measured the WC, but found that the biopsies containing tendon had very low WC (Figure 3). We therefore excluded these six biopsies from further analysis.

Total body water.

Deuterium oxide space (V-D₂O) was used to determine total body water (TBW). In order to determine V-D₂O, the principle of dilution was applied (the method is described in details elsewhere (38)):

\[ V-D_2O = (V_i - V_u)/(D_{plasma,420} - D_{plasma,0}) \]

where \( V_i \) = injected volume D₂O; \( V_u \) = D₂O volume in urine; \( D_{plasma,0} \) and \( D_{plasma,420} \) are fractional abundances of deuterium in the plasma at time 0 and time 420 min.

Data and Statistical analysis.
R version 2.10.1 was used for calculations and statistics (43). Data are presented as mean with standard deviations (SD), except in one case in table 1, where the median together with the 25th and 75th percentile are presented due to deviation from normality. Differences between groups were assessed by unpaired *t*-test or Mann-Whitney Rank Sum test and differences between methods and within animal were assessed using paired test. In order to analyze the repeated measurement hierarchical modeling was accomplished by using linear mixed effects (lme) in package non-linear mixed effects (nlme)(42). Plasma [Na⁺] from adjacent measured values was fitted to the scanning times using spline interpolation.

**Results**

*Changes in extracellular electrolytes and osmolality.*

In the present study, intravenous administration of hypotonic fluid and DDAVP caused progressive hyponatremia (Figure 4A). The plasma sodium concentration was reduced by 11% from 138 mmol/l (SD=1) to 123 mmol/l (SD=2) after 420 min. In parallel, the plasma chloride concentration was progressively reduced (from 101 mmol/l (SD=2) to 88 mmol/l (SD=2)) (Figure 4B) as was the plasma osmolality (from 291 mOsm/kg (SD=6) to 261 mOsm/kg (SD=5)) (Figure 4D). Thus, the plasma sodium concentration determined the plasma osmolality. This excludes substantial translocational hyponatremia with shift of water from the intracellular compartment to plasma caused by osmotic active substances other than Na⁺ confined to plasma. The plasma potassium concentration rose from 3.5 mmol/l (SD=0.2) to 3.9 mmol/l (SD=0.4). Cardiovascular dynamics were stable with a mean
arterial pressure of 96 mmHg (SD=12) and a heart rate of 74 beats per minute (SD=16). pO₂, pH and arterial CO₂ pressure were unchanged: 37.2 kPa (SD=12.1), 7.45 (SD=0.05) and 5.8 kPa (SD=0.9), respectively. Renal parameters are shown in Table 1.

_Acute hyponatremia causes brain and muscle edema._

The changes in brain and muscle WC during the 420-min period are shown in Figure 5. The data was analyzed with mixed models in order to capture the inherent dependency of the repeated measurements in each animal. In all brain regions (whole brain (Figure 5A), thalamus (Figure 5B) and white matter (Figure 5C)) and in the masseter muscle (Figure 5D), the increase in WC calculated from the T₁ maps was higher in the hyponatremic animals than in the controls. Figure 6 shows an example of cerebral tissue compression following the development of brain edema in acute hyponatremia. In all animals at baseline the WC in whole brain was 341 g water per 100 g dry weight (dw) (SD=18), in thalamus 275 g water per 100 g dw (SD=17), and white matter 217 g water per 100 g dw (SD=9).

The WC estimated by the T₁ maps increased in a heterogeneous manner in the different brain regions. In the whole brain the WC rose by 6% (P<0.01), whereas the rise in the area of thalamus was 12% (P<0.01), and in the relative water-free area of the white matter, it was only 3% (P=0.04) (Table 2). Furthermore, the WC rose by 11% in muscles (P<0.01) (Table 2).

In order to _localize_ the water content changes brain ADC maps were calculated. There was a tendency towards a decrease in ADC in the whole brain; however the decrease was not significant (P=0.09) (Table 3). In thalamus and white matter ADC did not change (Table 3).
Brain and muscle water content in relation to plasma [Na⁺].

In order to compare the changes in WC directly in different brain areas and muscle, the WC was normalized with the first measurement in every animal. The relation between normalized WC and plasma [Na⁺] was analyzed using linear mixed models and described by the slope coefficients (Table 4). In the thalamus and muscles, the WC change was about the same, but significantly less WC change was observed in the whole brain and white matter than in muscle and thalamus (P < 0.01).

To further analyze the relationship between WC and [Na⁺], we compared the WC increase that should be expected if the regions behaved as perfect osmometers with the actually determined coefficients for WC increases. The expected WC increase was calculated as the slope coefficient between the two points ([Na⁺]₀, 100) and ([Na⁺]₄₂₀, 100·([Na⁺]₀/[Na⁺]₄₂₀)), where [Na⁺]₀ is plasma [Na⁺] at time zero, [Na⁺]₄₂₀ is plasma [Na⁺] at time 420 min, 100 is the normalized WC at time zero, and 100·([Na⁺]₀/[Na⁺]₄₂₀) the normalized WC at time 420 if the WC changed proportionally with plasma [Na⁺]. The expected slope coefficient will obviously always be -100/[Na⁺]₄₂₀. Plasma [Na⁺]₄₂₀ is 123 mmol/l and the expected slope would therefore be -0.81 [Na⁺]⁻¹. Table 4 compares the experimentally determined and the expected coefficients. The WC increase in the whole brain was significantly less than expected (-0.47/-0.81 = 58%) and consistent with a WC increase of 6% (Table 2). The WC increase in white matter was even less, reaching only 31% of the expected increase (-0.25/-0.81).

Nevertheless, thalamus and muscle swelling were as expected from perfect osmotic behavior.

Brain sodium content decreases in acute hyponatremia.
The sodium content decreased significantly (11 µmol/100 g dw (dry weight) (confidence interval: 2-20, P=0.02)) in brains from hyponatremic animals compared with controls (Figure 7). Although the cerebral potassium content was higher in the hyponatremic animals (Figure 7) than in the non-hyponatremic animals, the difference was not significant (P=0.16).

No significant difference in the muscle content of sodium or potassium was observed between the hyponatremic group and the control group at time 420 min (Figure 8) (P=0.27 and P=0.91, respectively).

Cross validation of MRI determined water content.

To validate the MRI-method, the brain WC was measured using deuterium oxide (D₂O). At time 420 min, the D₂O-determined WC was 84.3 g water per 100 g wet weight (ww) (SD=1.3, n=7) compared with 78.0 g per 100 g ww (SD=1.1, n=7) with MRI (whole brain). The relation between MRI- and D₂O-determined WC is shown in Figure 9A.

The MRI method was cross-validated using muscle biopsies harvested at time zero and 420 min. The WC in the muscle biopsies was determined physically by lyophilization. The WC was 78.5 g water per 100 g ww (SD=2.3) in biopsies (n=24) when measured physically compared with 75.8 g water per 100 g ww (SD=1.6) when the MRI-method was used. The relation between the MRI-determined and the physically determined WC is shown in Figure 9B. Using both methods, the relative increase in WC was

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1 The reduction of Na⁺ of 11 µmol/g dw equals 3.3 µmol/g wet weight (ww) (WC is 70%). The pig brain ww is approximately 80 g and the pig brain contains 264 µmol sodium (80 g · 3.3 µmol/g ww). At the end of the experiment the plasma [Na⁺] was 123 mmol/l = 123 µmol/ml. The sodium loss can then be explained by squeezing out 2.1 ml of ECV (264 µmol sodium/123 µmol/ml), 3% of whole pig brain volume.
significant (P = 0.02 and P=0.04, respectively) and almost identical (10.52% and 11.14%, P = 0.915, paired test). Furthermore, no significant difference in muscle WC was observed between control animals at time zero and time 420 min.

Discussion

The study demonstrates that moderate hyponatremia causes brain edema in a large animal. The increase in WC was inversely proportional to plasma [Na\(^+\)] in thalamus and muscles. In slices of the whole brain and in white matter, the WC increase was less than should be expected, and a decrease in brain sodium content was observed.

*In vivo water measurement in an animal model correlates with in vitro measurements.*

Neurological disasters are common in acute hyponatremia. We therefore studied the effects on brain and muscle water in this condition. Because the time course is important, we used an infusion model previously described in pigs (38). The model is clinically relevant in the sense that it induces hyponatremia during a time span of hours compared with common methods in rodents which imply instant infusion of large quantities (20% of the animal’s weight) of sterile water into the peritoneum (2; 28; 29; 34) or into the stomach (5; 7). However, exceptions exist where acute hyponatremia is induced in rats over hours by administration of electrolyte-free water by gavage (55).

The control group was not treated with desmopressin in order to use the same model reported in a recent study from our group (38) and to achieve a large difference in plasma [Na\(^+\)] between groups.
The V₂-receptor agonist desmopressin was chosen to avoid a direct effect on the tissues of interest known with the combined V₁-V₂-receptor agonist vasopressin (46). Most studies agree that desmopressin only to a limited degree penetrate over the blood-brain barrier (51; 52). It would certainly also be of interest to investigate a model of pure hyponatremia in which both groups were treated with desmopressin and only the sodium concentration varied to clarify this issue. Likewise, it will be necessary to clarify the possible importance of a direct effect of vasopressin as compared to desmopressin on the brain (46).

MRI was used to capture WC changes in brain and muscle. Previous studies have shown that the MRI-determined brain WC is lower than the physically determined WC (34; 59). Consistent with these studies, the results of the present study showed a lower absolute WC in the slice of the whole brain at time zero than has been described in rodents (7; 31; 59). However, the WC showed little variation in all regions and expected differences in WC between the relatively water-sparse white matter compared with the area of the thalamus were measured.

To cross-validate the MRI-determined WC, the WC in brain was also determined using the dilution method with D₂O. The D₂O-measured brain water was higher than MRI-determined water.

There may be several reasons why MRI-derived WC estimations were lower than those found by the deuterium dilution and tissue drying methods:

1) Equilibrium constants may be different for binding of water to tissue proteins and to the gelatin standards used for calibration of the system (16).
2) Discrepancy might evolve from measuring WC in different regions with MRI and with physical methods. Whereas WC is calculated from T₁ maps of a slice of the brain with the MRI method, the deuterium method and the drying method used by others (7; 31; 59) are applied to the whole brain.

3) Higher WC determined with the D₂O method might also be result if the steady-state between brain water and plasma water is not achieved after eight hours, but we find this unlikely given the results of previous studies of water exchange between blood and tissues (14).

For further cross-validation we determined WC in muscle biopsies with lyophilization. This demonstrated higher WC than with the MRI method which may be ascribed to the before-mentioned possible differences in equilibrium constants and different blood content, even though the muscles were carefully dissected and wiped with a towel to remove external blood. Notwithstanding the reservation that applies to the exclusion of six biopsies, we did find proportionality between the WC increase measured with both methods.

The T₁ effect of increasing water content in tissue might differ according to tissue types. If the relaxivities differs substantially this might introduce an error in the water content estimation. However, the method for water content estimation applied in this study was calibrated specifically on brain white matter (16). Therefore, both the precision and accuracy in white matter water content estimation is expected to be high.

Passive brain water control in acute hyponatremia.
Acute hyponatremia induced brain edema in this pig model. This is consistent with the bulk of data from rodent studies (2; 5; 7; 22; 29; 34; 35) and case reports from patients (11; 13; 50). The WC rose 6% in the slice of the whole brain which is less than should be expected if the brain shows perfect osmotic behavior. Previous whole brain studies demonstrated a similar discrepancy between measured and expected water increase (7; 29; 65). The explanation may partly be rooted in the fact that the whole brain, i.e. including white matter, gray substance, ventricles and blood, was investigated together with the slice of the brain in our study, whereas in rodent studies, WC is determined physically in the whole brain. We found that white matter (in all animals at baseline) contained significantly less water than the whole brain. Further, the water increase in white matter was less than that seen in the whole brain. The whole brain contains white matter which can explain some of the discrepancy between measured and expected water increase. Another possible explanation is the existence of cerebrospinal fluid (CSF) (ventricles, sulci) and blood in the whole brain. A reduction of these water filled spaces when the cells are swelling in the rigid scull would be measured as if the WC in the whole brain did not increase as much as expected.

In order to examine why the water increase was less than expected, we measured the potassium and the sodium content in the whole brain. The brain sodium content in hyponatremic animals was reduced, whereas the potassium content was the same in control and hyponatremic animals. This is consistent with data reported in acute hyponatremia in rodents (7; 22; 29) and cat (65). Interestingly, we observed a reduction in the extracellular ion sodium and not in the intracellular ion potassium which we would have expected if RVD should explain the discrepancy between measured and expected WC after 420 min. A simple explanation would be that the majority of brain cells swell in acute hyponatremia and sodium containing extracellular water (CSF and blood) is squeezed out of the rigid scull according to
the Monro-Kellie doctrine (23; 32). This mechanism has previously been proposed (29; 40; 54).

Extrusion of sodium from the brain along with ECV is supported by the fact that the WC in the area of
the thalamus increases inversely proportional to plasma [Na\(^+\)] content; i.e., cells in this region show
perfect osmotic behavior as very little extracellular water is moved from this region when cells swell.
This is consistent with the finding of no RVD in cortical brain cells studied in brain slices (3). The
findings support the view that brain edema is of primary importance in the symptomatology of acute
hyponatremia; however, RVD not detectable with MRI could cause an increase in the extracellular
concentration of excitatory neurotransmitters. This could contribute to the causation of seizures (64)
and must be investigated further. That potassium content does not decline in this study is in contrast to
a study in rats where plasma [Na\(^+\)] was reduced to 106 mmol/l within seven hours(55). The difference
might be due to the lower plasma [Na\(^+\)] in this study or the use of vasopressin to induce the antidiuretic
state in the rats compared to desmopressin in our study. Studies with more severe acute hyponatremia
in large animal must be performed to elucidate the issue and also studies comparing vasopressin with
desmopressin.

Still, in the study of the intact organ in vivo, water was measured in the tissue containing both ECV and
ICV. This may explain some of the discrepancies between our study and cell studies in anisotonic
solutions which have shown almost immediate RVD (21; 36). In order to describe the relationship
between ICV and ECV ADC maps were calculated. ADC showed a decreasing tendency in the whole
brain (P=0.09). This can be explained from the decrease in the ECV (CSF and blood is squeezed out of
the scull) relative to an increase in the ICV (overall limited RVD in the brain) (12; 18; 49). ADC did
not change in thalamus or white matter (P=0.26 and 0.61, respectively). This can be attributed to a
relative increase in both ICV and ECV so the fraction of ICV/ECV did not change. In thalamus where a
large increase in WC (12 %) is observed this is consistent with limited RVD as both ICV and ECV is thought to increase in this dilution model of hyponatremia. Extensive RVD would tend to increase ADC as the ICV/ECV fraction would decrease. In white matter where a lower increase in WC is observed (3 %) the ADC did not change. This could be caused simple by the change being too small to result in measurable changes in the ICV/ECV fraction. If massive RVD should explain the non-osmotic behavior of white matter we would expect an increase in ADC caused by a lower ICV/ECV fraction. Another explanation for the small WC increase and unchanged ADC might be slow water equilibration in white matter with proportional increase in ICV and ECV. However, ADC is a measure of the diffusion by the random Brownian motion of protons. Therefore ADC does not only depend on the ICV/ECV fraction but also water molecules in different physiochemical states. Main factors would be macromolecular water binding, molecular crowding, microviscosity and compartmentation, including microcompartments within the cells and between cells caused by differences in membrane permeability (47; 56). Therefore, the ADC results must be interpreted cautiously. Altogether, the ADC results agree with limited RVD in the whole brain and thalamus and put some reservation on the findings in white matter to indicate RVD. We still want to stress the possibility, however, of RVD in this area based on the T1 data because of the possible link with osmotic demyelinization with a predilection in white matter.

Crucially, we studied all the cells in the region (complex mix of neurons and neuroglial cells) and were unable to distinguish the differences among neurons and neuroglial cells shown in cell studies (1; 3; 36; 40). Recent studies of astrocytes in vivo show these cells to react passively to osmotic challenges with no signs of RVD corresponding with the findings in this study (33; 45). Since previous studies (3) indicated that “the neuronal compartment is osmotically water-tight” (45) perhaps because of the
absence of aquaporins, the studies on astrocytes raise important questions with regard to the nature of brain edema in hyponatremia. Perhaps we may envision that “the neuronal compartment” mainly swells secondary to cytotoxic effects be it secondary to hypoxia or ischemia induced by pressure from the overcongested “astroglial compartment”. Since astrocytes apparently do not undergo RVD in the cited in vivo studies, RVD might occur specifically in the neurons and hence require previous neuronal jeopardy. Hence in comparison to the study in which brain potassium declined (55) we suggest that the specificities of that study did in fact cause sufficient neuronal injury and swelling to induce delayed RVD.

However, this leaves us with the problem of explaining one main finding in the study, i.e. the passive and apparently perfect osmotic behavior in thalamus which can hardly be ascribed only to astrocytes swelling in hyponatremia. We can probably assume that also neurons were engaged and that the duration of the study was too short or that the degree or acuteness of hyponatremia too limited to cause apparent RVD.

Another difference between our study and cell studies is the gradual decrease in osmolality in the pig compared with the instantaneously changed osmolality in cell studies, and the fact that the cells studied in vitro are suspended in a large volume of solutes very unlike the delicate composition of CSF, and the fact that cells are not surrounded by neural elements. All these factors are likely to influence the results. The non-osmotic behavior of white matter can be caused by RVD or it may simply occur because of slow water equilibrium in the hydrophobic region. Extensive RVD in oligodendrocytes has been linked with osmotic demyelination (OD) (24); however, simple differences in water equilibrium between the axon and the myelin-containing oligodendrocyte and thereby differences in volume changes may also destroy the delicate interaction between the two cells and contribute to OD.
**Homeostatic behavior of muscles in acute hyponatremia.**

The masseter muscle shows perfect osmotic behavior in moderate, acute hyponatremia. Further, no significant electrolyte changes were observed in muscle homogenates. The observation of perfect osmotic behavior in skeletal muscles is consistent with the findings in a study of single muscles fibers (10) and in studies on isolated muscles (19; 39). However, others have produced evidence of RVD in isolated muscle cells (17; 26; 37) and less WC in isolated rat muscle than should be predicted from the change in osmolality (4).

Muscle WC was measured repeatedly in its normal environment (anatomic structures, hormones and cytokines) and in a clinically relevant model of acute hyponatremia induced over hours. The study therefore adds knowledge to single cell and isolated muscle studies. When treating or preventing acute hyponatremia in the clinic, it is important to take the entire system into account. In the clinic, muscles are in their natural environment and because they contain 50% of TBW and 70% of total body potassium, their response to hyponatremia is extremely important (58). This study demonstrates that the WC in the masseter muscle is inversely proportional to the plasma $[\text{Na}^+]$, wherefore acute RVD is unlikely to happen in the muscle in acute hyponatremia in our model. This is consistent with a previous study using the same model where plasma $[\text{Na}^+]$ was predictable from external balances of water and electrolytes according to Edelman’s equations (38). Extensive RVD in the muscle with efflux of potassium and organic osmolytes would hamper the logic of Edelman’s relation where plasma sodium is determined by exchangeable $\text{Na}^+$ (eNa$^+$), exchangeable $\text{K}^+$ (eK$^+$), and TBW (15). Without changing eNa$^+$, eK$^+$ or TBW, acute RVD would decrease plasma $[\text{Na}^+]$ more than expected because of
translocational hyponatremia (38). In support of this, it is the plasma \([Na^+]\) that determines plasma osmolality in this model (Figure 4A and 4D), and no changes in the muscle content of potassium and sodium in the time frame of 420 min were recorded (Figure 8). In addition to this, we observed no increase in plasma \([K^+]\) (Figure 4C), nor was the excretion of potassium in the urine elevated in the hyponatremic animals (Table 1). The time frame in acute hyponatremia is undoubtedly important in regard of the organism’s water and electrolyte regulation. One might speculate that in the intact organism muscle RVD has a slow onset so that lethal hyperkalemia is avoided, and that the RVD continues slowly over hours/days. This is consistent with Verbalis’ elegant studies in rats with continued hyponatremia for several days (61; 62), where a significantly increased potassium excretion in urine from day one and forth was observed, corresponding with a decrease in whole-body potassium. Because the bulk of potassium is situated in the muscles, it is reasonable to speculate that the potassium is lost from the muscles in conjunction with RVD.

The observed reduction in extracellular \([Cl^-]\) (Fig 4B) may have effects on the function of the skeletal muscle and neurons during hyponatremia. Thus, it has recently been shown that a reduction in the \(Cl^-\) conductance increases the excitability of muscle fibers (41). Similarly, decreases in \(Cl^-\) conductance due to channelopathy in the predominant muscular \(Cl^-\) channel (ClC1) as seen in congenital myotonia leads to delayed muscular relaxation (25). The decrease in extracellular \(Cl^-\) concentration present during hyponatremia may increase the excitability, thereby exacerbating or even being partly responsible for the seizures often seen in acute hyponatremia (5; 53). This tentative link, however, needs further study.
Conclusions and perspectives.

This *in vivo* study demonstrates that acute moderate hyponatremia causes brain edema in a large animal. Extensive RVD does not occur in the cell-rich thalamus or in the main part of brain during seven hours of hyponatremia. However, acute RVD or slow water equilibrium in white matter are possible and should be further investigated. This corresponds with the predilection of oligodendrocytic destruction in osmotic demyelination seen when plasma sodium is corrected too quickly in the hyponatremic patient. Future studies of a causal connection are needed. However, the results support the clinical consensus that symptoms in acute hyponatremia are primarily caused by brain edema, and the treatment of choice is alleviating the brain edema with a bolus of hypertonic NaCl (53). Next, if the non-perfect osmotic behavior of the white matter is related to osmotic demyelination, one must be extremely careful not to elevate plasma [Na$^+$] too quickly after the initial treatment of the edema, not only in the chronic cases but also in acute hyponatremia. This is also in accordance with existing treatment guidelines (53; 63).

The lack of acute RVD in the skeletal muscle, which contains the bulk of the organism’s water and potassium, is important with regard to treatment and the whole-body response to acute hyponatremia. This finding suggests that the muscle mass acts as a buffer in acute hyponatremia. Active volume regulation with efflux of potassium and organic osmolytes could: 1) increase plasma [K$^+$] dangerously; 2) cause further translocational decrease in plasma [Cl$^-$], result in increased excitability of muscles and neurons and thereby enhance the risk of seizures; and 3) cause further decrease in plasma [Na$^+$]. However, future *in vivo* electrophysiological studies must investigate this together with the response to more sustained hyponatremia.
Acknowledgements

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No conflict of interest.
Appendix

Calculation of brain water using deuterium

\( V_{\text{total}} \): Total volume of CNS and added TCA

\( V_{\text{cns}} \): CNS volume

\( V_{\text{tca}} \): Added TCA volume

\( D_{\text{tca}} \): fractional abundance of deuterium (ppm) in the added TCA

\( D_{\text{sol}} \): fractional abundance of deuterium (ppm) in the solution after equilibrium

\( D_{\text{plasma,420}} \): fractional abundance of deuterium (ppm) in plasma at time 420 min

Eq 1: \( V_{\text{total}} = V_{\text{cns}} + V_{\text{tca}} \)

Eq 2: \( V_{\text{total}} \times D_{\text{sol}} = V_{\text{cns}} \times D_{\text{plasma,420}} + V_{\text{tca}} \times D_{\text{tca}} \)

(On the assumption that plasma water and brain water are in steady state, so \( D_{\text{plasma,420}} = D_{\text{CNS,420}} \))

Substituting \( V_{\text{total}} \) in Eq 2 with Eq 1 gives

\( D_{\text{sol}} \times (V_{\text{cns}} + V_{\text{tca}}) = V_{\text{cns}} \times D_{\text{plasma,420}} + V_{\text{tca}} \times D_{\text{tca}} \)

\( D_{\text{sol}} \times V_{\text{cns}} + D_{\text{sol}} \times V_{\text{tca}} = V_{\text{cns}} \times D_{\text{plasma,420}} + V_{\text{tca}} \times D_{\text{tca}} \)

\( D_{\text{sol}} \times V_{\text{cns}} - V_{\text{cns}} \times D_{\text{plasma,420}} = V_{\text{tca}} \times D_{\text{tca}} - D_{\text{sol}} \times V_{\text{tca}} \)

\( V_{\text{cns}} = V_{\text{tca}} \times (D_{\text{tca}} - D_{\text{sol}})/(D_{\text{sol}} - D_{\text{plasma,420}}) \)
Reference List


Figure legends

Figure 1. Imaging protocol. Investigation of the effect of hyponatremia on brain and muscle water in the anaesthetized pig by magnetic resonance imaging (MRI). The set of MR-sequences was repeated seven times (MR1-MR7). Each scanning session included $T_1$ weighted sequences, inversion recovery sequences for $T_1$ estimation and diffusion weighted recordings. The design was identical in the two groups of animals (Hyponatremia +/-).

Figure 2. Representative picture of the brain and muscle section with regions of interest (ROI). ROI no.1, whole brain. ROI no.2, thalamus. ROI no.3, white matter. ROI no.4, masseter muscle.

Figure 3. Muscle water determined in lyophilized biopsies. Biopsy harvested at time zero is linked with the biopsy from the same animal at time 420 min. Biopsies marked with circles is excluded, because of abnormal water content (plain text for details).

Figure 4. Plasma concentration of $Na^+$ (A), $Cl^-$ (B) and $K^+$ (C). Plasma osmolality (D). Filled black circles (•), hyponatremic animals; unfilled circles (o), control animals; Broken, vertical, gray line (– –), intervention begins.
**Figure 5.** Water content in Whole brain (A); Thalamus (B); white matter (C); Masseter muscle (D). Filled black circles (•), hyponatremic animals; unfilled circles (o), control animals; dw, dry weight.

**Figure 6.** Example demonstrating the development of cerebral edema following acute hyponatremia (Left, before reduction of plasma [Na⁺]; right, after seven hours of hyponatremia). The brain is pressed towards the basis of the cranium (A); the ventricles have been compressed (B); and, finally, the sagittal sinus has been compressed (C).

**Figure 7** Brain sodium content (A) and brain potassium content (B) in control (n=4) and hyponatremic animals (n=3). (*) indicates significantly difference (P<0.05). Black line (—), mean value; dw, dry weight.

**Figure 8.** Muscle sodium content (A) and muscle potassium content (B) in control (n=7) and hyponatremic animals (n=6). Black line (—), mean value; dw, dry weight.

**Figure 9.** Cross-validation of MRI determined water content with D₂O (brain) and lyophilization (muscle) determined. A: MRI versus D₂O determined water content (WC) in brain (n=7). B: MRI versus lyophilization to determine WC in muscles (n=24). Black line (—), line of identity; ww, wet weight.
Table 1 Renal parameters. Excretion of urine and electrolytes as well as estimation of total body volume.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Hyponatremic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>7</td>
</tr>
<tr>
<td>UVol, l/seven hours</td>
<td>0.3 (0.1)</td>
<td>0.4 (0.2)</td>
</tr>
<tr>
<td>UVol, ml/kg/hour</td>
<td>1.3 (0.6)</td>
<td>1.5 (0.6)</td>
</tr>
<tr>
<td>UNa· UVol, mmol/kg/hour</td>
<td>0.08 (0.1)</td>
<td>0.07 (0.07)</td>
</tr>
<tr>
<td>UK· UVol, mmol/kg/hour</td>
<td>0.11 (0.05)</td>
<td>0.07 (0.03)</td>
</tr>
<tr>
<td>UCreatinine· UVol, µmol/kg/hour</td>
<td>0.013 (0.003)</td>
<td>0.015 (0.002)</td>
</tr>
<tr>
<td>Uurea· UVol, mmol/kg/hour</td>
<td>0.21 (0.09)</td>
<td>0.23 (0.13)</td>
</tr>
<tr>
<td>Uosm, mOsm/kg water</td>
<td>619 (456-695)</td>
<td>641 (397-752)</td>
</tr>
<tr>
<td>TBW420, l</td>
<td>25.9 (1.2)</td>
<td>31.3 (1.6)</td>
</tr>
</tbody>
</table>

Data are means followed by standard deviation (SD) in brackets, except U_{osm} where data are medians followed by the 25 and 75 percentile. UVol, urine volume; UNa, Urine sodium; UK, urine potassium; UCreatinine, urine creatinine; Uurea, urine urea; U_{osm}, urine osmolality; TBW420, total body water time 420 min.
Table 2 Water content in brain regions and muscle in hyponatremia

<table>
<thead>
<tr>
<th>Water content / 100 g dw</th>
<th>Time zero</th>
<th>Time 420 min</th>
<th>Difference</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole brain</td>
<td>342 (SD=14)</td>
<td>363 (SD=21)</td>
<td>6%</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Thalamus</td>
<td>277 (SD=13)</td>
<td>311 (SD=24)</td>
<td>12%</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>White matter</td>
<td>219 (SD=7)</td>
<td>225(SD=5)</td>
<td>3%</td>
<td>P=0.04</td>
</tr>
<tr>
<td>Muscle</td>
<td>304 (SD=19)</td>
<td>338 (SD=33)</td>
<td>11%</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Plasma [Na⁺]</td>
<td>138 (SD=1)</td>
<td>123(SD=2)</td>
<td>-11%</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

Water content in different brain regions and in muscle of hyponatremic animals. Data are means followed by standard deviation (SD) in brackets. Dw, dry weight; Difference, relative difference in water content from time zero to time 420 min; P-value, paired-test between water content at time zero and time 420 min.
Table 3 Apparent diffusion coefficient of water in hyponatremia

<table>
<thead>
<tr>
<th></th>
<th>Time zero</th>
<th>Time 420 min</th>
<th>Difference</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole brain</td>
<td>951 (38)</td>
<td>925 (52)</td>
<td>-3%</td>
<td>0.09</td>
</tr>
<tr>
<td>Thalamus</td>
<td>814 (59)</td>
<td>844 (79)</td>
<td>+4%</td>
<td>0.26</td>
</tr>
<tr>
<td>White matter</td>
<td>727 (61)</td>
<td>739 (79)</td>
<td>+2%</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Apparent diffusion coefficients (ADC) \(10^{-12} \text{ m}^2/\text{s}\) in different brain regions of hyponatremic animals. Data are means followed by standard deviation (SD) in brackets. Difference, relative difference in ADC from time zero to time 420 min; P-value, paired-test between ADC at time zero and time 420 min.
Table 4 Normalized water content in relation to plasma [Na⁺]

<table>
<thead>
<tr>
<th>Region</th>
<th>Coefficient</th>
<th>P₁-value</th>
<th>P₂-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole brain/[Na⁺]</td>
<td>-0.47 (SE=0.07)</td>
<td>P&lt;0.01</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Thalamus/[Na⁺]</td>
<td>-0.87(SE=0.13)</td>
<td>P=0.45</td>
<td>P=0.66</td>
</tr>
<tr>
<td>White matter/[Na⁺]</td>
<td>-0.25(SE= 0.09)</td>
<td>P&lt;0.01</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Muscle/[Na⁺]</td>
<td>-0.86(SE=0.06)</td>
<td>P=1</td>
<td>P=0.75</td>
</tr>
<tr>
<td>Perfect osmotic behavior</td>
<td>-0.80 (SE=0.03)</td>
<td>P=0.75</td>
<td>P=1</td>
</tr>
</tbody>
</table>

Normalized water content in different brain regions and muscle, in relation to plasma [Na⁺].

Coefficient, slope coefficient from the linear mixed model analysis; SE, standard error; Perfect osmotic behavior, the slope coefficient for a tissue where water increase proportionally with plasma [Na⁺] (simulated); P₁-value, t-test between muscle slope coefficient and the slope coefficient of the region; P₂-value, t-test between slope coefficient in tissue with perfect osmotic behavior (simulated) and the slope coefficient of the region of interest.
MR1  MR2  MR3  MR4  MR5  MR6  MR7  Scanning

-70  0  70  140  210  280  350  420 Minutes

Hyponatremia +/-
Hyponatremic animals

Muscle water, g water per 100 g dw

Control animals

Hyponatremic animals