Urea minimizes brain complications following rapid correction of chronic hyponatremia compared with vasopressin antagonist or hypertonic saline

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Hyponatremia is a common electrolyte disorder that carries significant morbidity and mortality. However, severe chronic hyponatremia should not be corrected rapidly to avoid brain demyelination. Vasopressin receptor antagonists (vaptans) are now being widely used for the treatment of hyponatremia along with other alternatives like hypertonic saline. Previous reports have suggested that, in some cases, urea can also be used to correct hyponatremia. Correction of severe hyponatremia with urea has never been compared to treatment with a vaptan or hypertonic saline with regard to the risk of brain complications in the event of a too rapid rise in serum sodium. Here, we compared the neurological outcome of hyponatremic rats corrected rapidly with urea, lixivaptan, and hypertonic saline. Despite similar increase in serum sodium obtained by the three drugs, treatment with lixivaptan or hypertonic saline resulted in a higher mortality than treatment with urea. Histological analysis showed that treatment with urea resulted in less pathological change of experimental osmotic demyelination than was induced by hypertonic saline or lixivaptan. This included breakdown of the blood–brain barrier, microglial activation, astrocyte demise, and demyelination. Thus, overcorrection of hyponatremia with urea resulted in significantly lower mortality and neurological impairment than the overcorrection caused by lixivaptan or hypertonic saline.

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Osmotic demyelination syndrome (ODS) is the most feared complication during the treatment of hyponatremia. It typically occurs few days after the too rapid correction of hyponatremia and is characterized by evidence of demyelination in the basal ganglia, subcortical areas, and the pons. The clinical signs are variable depending on the demyelinated tracts involved.[1,2] The magnitude of increase of serum sodium (SNa) in 24 h is the most important risk factor of osmotic demyelination, but that increase in SNa depends on many variables and is poorly predictable during hyponatremia correction.[3] The sequence of events leading to demyelination after rapid correction of severe hyponatremia is still not well understood. During chronic hyponatremia, a significant decrease in the content of brain organic osmolyte contributes to the osmotic buffering of the brain to prevent edema; when chronic hyponatremia is corrected rapidly, the re-accumulation of these osmolytes is slow[4,5] and the delay in the reaccumulation of these small molecules might play a part in the events leading to ODS. Recently, we have shown that astrocyte death occurs very early in ODS and is accompanied with a loss of astrocyte–oligodendrocyte gap junctions,[6] which may compromise astrocyte trophic support to oligodendrocyte, integrity of the blood–brain barrier (BBB), and also induce microglial activation. Indeed, the well-defined histological hallmarks of ODS are BBB breakdown, microglial activation, and demyelination. Several vasopressin receptor (V2R) antagonists have been approved for the correction of euvolemic and hypervolemic hyponatremia around the world.[7] These molecules called vaptans act by inhibiting vasopressin-dependent translocation of the water channel at the lumen of the distal circonvoluted tubule and therefore water reabsorption; these events will induce electrolyte-free diuresis and increase the SNa levels. Although in most of the studies with vaptans the reported increment of SNa is <10 mEq/l/24 h, there is a risk of a higher increase in SNa,[8,9] and a recent meta-analysis showed that the risk of an increase of serum sodium of >10 mEq/l/day with vaptans is around 10%.[10] Preliminary animal studies have suggested that vaptans could carry some risk of ODS.[11,12] However, so far only one case of human ODS has been published in which the use of vaptans unfortunately led to an excessive

correction gradient (66 mEq/l over 72 h) along with hyperosmolarity.[13] Another option for the treatment of hyponatremia is urea, and our team has reported that urea is effective in correcting euvolemic and even hypervolemic hyponatremia.[14,15] Interestingly, urea, through an unclear mechanism, acts as a cryoprotectant and an osmoprotectant in amphibians.[16,17] It has also been reported that urea could protect rat heart during electrolysis and increase murine kidney cell viability after hyperosmotic stress.[18,19] Our previous experimental studies suggested that rapid correction of severe hyponatremia with urea might carry a lower risk of neurological complications when compared with treatment with hypertonic saline (HS).[20,21] The protection afforded by treatment with urea seems to be independent of the kinetics of the other major organic osmolyte re-accumulation during hyponatremia correction.[20] It remains, however, unknown whether correction of hyponatremia with urea or vaptans is accompanied by any significant changes on the histological hallmarks of osmotic demyelination such as BBB rupture, microglial activation, and astrocyte loss. We wanted to investigated the effects of overcorrection of hyponatremia with urea, HS, and a V2R antagonist on an experimental model of ODS.

RESULTS

Compared with HS, both urea and lixivaptan are effective in correcting chronic hyponatremia in rats

Administration of a liquid diet with vasopressin infusion resulted in hyponatremia in all the six studied groups of rats (see Supplementary Figure S1 online for experimental design and Table 1 for biochemical parameters). Mean SNa values after the induction of hyponatremia ranged from 108 to 114 mEq/l. In all groups, there was a sharp increase of the SNa 24 h after the initiation of the treatment (32–33 mEq/l), regardless of the experimental paradigm.

Fractionated doses of HS or lixivaptan induced a similar twelfth hour SNa increment compared with urea (16 ± 1, 18 ± 1 and 19 ± 1 mEq/l respectively, Table 1 and Figure 1). Blood level of urea was higher in animals that received urea compared with animals treated with lixivaptan or HS.

Mortality and neurological manifestations associated with overcorrection of hyponatremia are significantly less when urea is used for correction than when lixivaptan or HS was used for correction

At 24 h after the beginning of the correction of hyponatremia, animals treated with lixivaptan or HS started to show neurological manifestations described in experimental ODS (lethargy, seizures, paralysis, and coma or death). Fewer animals treated with urea displayed those signs, which were less marked than in animals treated with V2R antagonist or HS (Figure 2a, $P<0.01$ for neurological score urea vs. lixivaptan or HS). In experiment 1, 6 days after the beginning of the correction of SNa, the mortality in the group of animals treated with urea was only 27 vs. 65 and 76% of animals treated with lixivaptan and HS, respectively ($P=0.017$ by Fischer's exact test—Table 1). Survival curve analyses (Figure 2c) confirmed survival benefit of treatment with urea vs. a bolus of lixivaptan or HS ($P=0.003$ by log-rank test).

The serum sodium increment after the first 12 h does not account for the effect of urea in ODS

Although the magnitude of SNa increase at 24 h is the main determinant of ODS,[1,2,22–25] earlier work has suggested that

Table 1 | Biological parameters of animals in experiments 1 and 2

	After the start of the correction										
	0 h		12 h		24 h		SNa increment		Outcome		
	SNa	Urea	SNa	Urea	SNa	Urea	12 h	24 h	Dead	Alive	Mortality
Experiment 1											
Group 1 $n=23$, lixivaptan once	112 ± 1	23 ± 2	NA	NA	145 ± 1	71 ± 9	NA	33 ± 1	15	8	65%
Group 2 $n=22$, urea 4 doses	108 ± 1	31 ± 1	NA	NA	141 ± 2	143 ± 30	NA	33 ± 1	6	16	27%
Group 3 $n=25$, NaCl single bolus	109 ± 1	28 ± 3	NA	NA	140 ± 1	45 ± 6	NA	31 ± 1	19	6	76%
Experiment 2											
Group 4 $n=17$, lixivaptan 2 doses	112 ± 1	29 ± 3	131 ± 3	42 ± 4	145 ± 2	52 ± 5	19 ± 2	33 ± 2	12	5	71%
Group 5 $n=16$, urea 4 doses	114 ± 1	22 ± 1	133 ± 1	158 ± 30	147 ± 1	187 ± 40	18 ± 1	32 ± 1	4	12	25%
Group 6 $n=15$, NaCl 2 doses	112 ± 2	27 ± 1	128 ± 1	27 ± 2	145 ± 2	44 ± 3	16 ± 1	32 ± 1	14	1[a]	93%

Abbreviations: NA, not applicable; SNa, serum sodium.
In the first experimental paradigm (Experiment 1), lixivaptan or hypertonic saline was administered in a single intraperitoneal (ip) bolus and urea in four divided doses. SNa was measured before the correction (0 h) and 24 h after the beginning of the correction. Groups 1–3 had similar increases in their Na at 24 h. ($P<0.001$ for SNa before and after in all the groups by paired *t*-test and $P=$ NS for increment in Na between all the groups by analysis of variance (ANOVA) test.)
In the second experimental paradigm (Experiment 2), lixivaptan or hypertonic saline was administered ip in two doses 12 h apart and urea ip in four doses every 6 h with Na measured at 0, 12, and 24 h. The three treatments induce a similar increment in Na at 12 and 24 h ($P=$ NS between the three groups by ANOVA both at 12 and 24 h).
In experiments 1 and 2, animals receiving urea had a lower mortality than animals receiving lixivaptan ($P=0.017$ and 0.015, respectively, by Fischer's exact test) or hypertonic saline ($P=0.0012$ and 0.0002, respectively). No difference in mortality was found in animals receiving lixivaptan vs. hypertonic saline in both experiments ($P=0.529$ and 0.178, respectively).
SNa = serum sodium in mmol/l and urea in mg/dl (to convert to blood urea nitrogen, multiply by 0.357). Range for urea groups 2 and 5 at 24 h: 68–620 and 79–590 mg/dl, respectively.
[a]The animal was alive early on day 10 and died later on the same day.

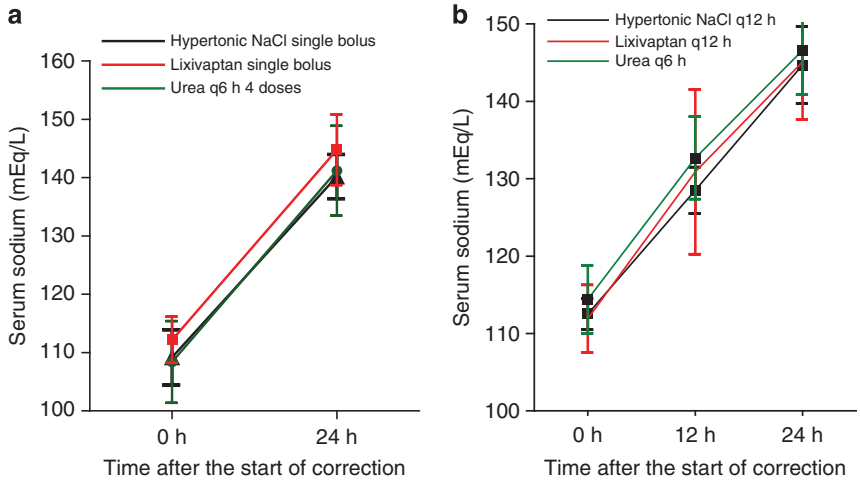

Figure 1 | Increment of serum sodium after the correction of hyponatremia with lixivaptan, hypertonic saline (HS), and urea. (**a**) In experiment 1, serum sodium was corrected with a single intraperitoneal (ip) dose of lixivaptan, HS, or four divided ip doses of urea. No difference in the increment of serum sodium at 24 h after the correction started in the three groups was seen. (**b**) In experiment 2, serum sodium was corrected with two doses of ip lixivaptan 12 h apart, two doses of ip HS, or four divided ip doses of urea (q6 hours). No differences in the increment of serum sodium in the three groups were seen 12 and 24 h after the beginning of sodium correction. $P = 0.01$ for serum sodium before and after the beginning of the correction in the three groups and both experiments. $P =$ NS for the overall sodium increment 12 h and 24 h after the beginning of the correction in the three groups in experiment 2 and at 24 h after the beginning of the correction in experiment 1.

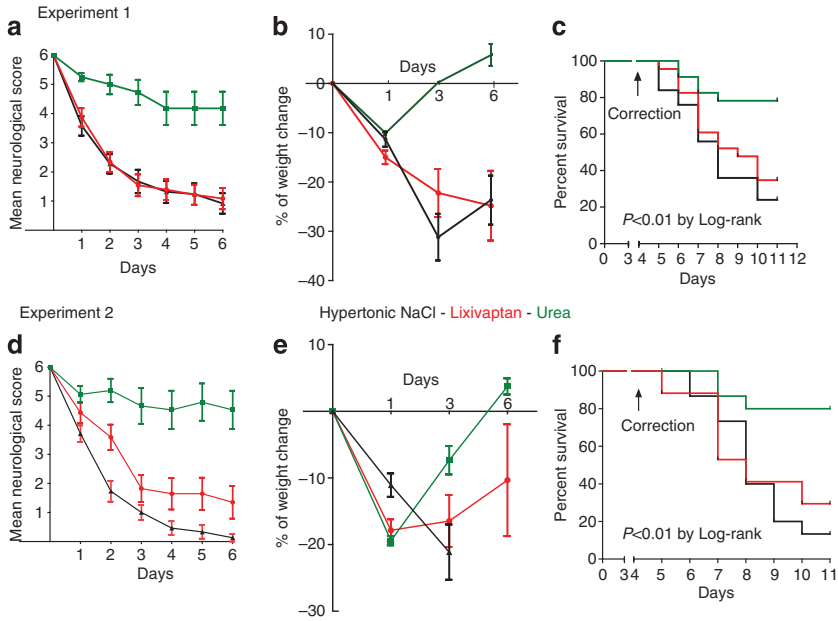

Figure 2 | Morbidity and mortality after the correction of hyponatremia with a vasopressin antagonist, hypertonic saline (HS), and urea. In experiment 1, we compared mortality in animals corrected with a single intraperitoneal (ip) bolus of either lixivaptan or HS with rats corrected with ip urea q6 hours. (**a**) From day 1 to day 6 after the beginning of hyponatremia correction, animals treated with urea displayed less severe neurological manifestations than animals corrected with lixivaptan or HS ($P = 0.01$ for urea vs. lixivaptan or HS from days 1 to 6 after the start of the correction by analysis of variance (ANOVA) test, $n = 20$–25). (**b**) Weight changes were similar 1 day after the correction of hyponatremia started in all groups, whereas 3 and 6 days after the initiation of the correction animals receiving urea had much less weight decrease as opposed to animals corrected with lixivaptan or HS ($P =$ NS for weight loss 1 day after the correction of hyponatremia and $P < 0.05$ at 3 and 6 days after the correction of hyponatremia, $n = 4$–12). (**c**) Finally, correction of hyponatremia with urea is associated with lower mortality than with a single bolus of either lixivaptan or HS. In experiment 2, both HS and lixivaptan were given in two doses q12 hours to achieve the same kinetic of serum sodium increase than with urea given at 4 doses q6 hours. Again, animals treated with lixivaptan or HS had a (**d**) lower neurological score and (**e**) continued losing weight compared with animals treated with urea ($P = 0.01$ from days 2 to 6 by ANOVA test, $n = 15$–17, and $P =$ NS for weight loss 1 day after the correction of hyponatremia began and $P < 0.05$ at 3 and 6 days after the correction of hyponatremia began. $n = 4$–17). (**f**) Despite the same kinetics in serum sodium increase, treatment with either lixivaptan or hypertonic saline was still associated with higher mortality compared with correction of hyponatremia with urea ($P < 0.01$ by log-rank test).

the rate by which the final increment is obtained might also have a role.[26] In clinical practice, it is observed that most of the diuresis afforded by the vasopressin receptor antagonists occurs within few hours after the administration of the drug.[7,27] Similarly, administration of a bolus dose of HS produces a brisk correction of hyponatremia, whereas on the other hand administration of urea in divided doses results in a more even diuresis during the entire administration period. In preliminary studies, we observed that 77 and 80% of the final increment of SNa was reached by HS and lixivaptan given as a bolus, whereas only 44% of the final increment is achieved at 12 h after the administration of urea (Supplementary Figure S2 online). It is therefore possible that the difference in the SNa increase accounted for the better outcome in the urea-treated group. To address that issue, we set up a second experimental design (experiment 2, Supplementary Figure S1 online) to achieve a similar correction gradient in the two groups 12 and 24 h after the treatment initiation. This was successfully accomplished by administering both lixivaptan and HS in two divided doses (Figure 1b and Table 1). Despite the same increment of SNa in the first 12 h, neurological manifestations were again worse and more frequent in animals receiving lixivaptan or HS than in animals given urea (Figure 2d, $P<0.01$ between the urea vs. HS or lixivaptan for neuroscore).

In experiment 2, the mortality in the urea-treated group was lower than in animals that received lixivaptan or HS (25, 71, and 93%, respectively. $P=0.015$ by Fischer's exact test for urea vs. lixivaptan and 0.0001 for urea vs. HS. $P<0.005$ by log-rank test for survival; Table 1, Figure 2f).

The overall mortality after HS or lixivaptan was similar regardless of the administration schedule (Supplementary Figure S3 online).

Compared with neuroscore, weight loss is a less biased assessment of the animal's impairment and is correlated with the neurological manifestations of ODS. We therefore compared the relative weight changes in animals treated with HS, urea, and lixivaptan in experiments 1 and 2 and found that all groups displayed the same magnitude of weight loss 24 h after the beginning of treatment. However, animals treated with urea started to gain back weight and showed a positive weight balance 6 days after the treatment initiation, whereas animals treated with lixivaptan or HS continued to lose weight and had a negative weight balance 6 days after the beginning of correction (Figure 2b and e, $P<0.05$ and <0.01 for weight changes in both experiments for 3 and 6 days after the treatment, respectively).

Both urea and lixivaptan can alleviate the changes in the permeability of the BBB in ODS

The rupture of the BBB is a hallmark of ODS.[6,28] We wanted to investigate the effects of lixivaptan and urea compared with HS on the BBB integrity through Evans blue (EB) dye analysis and immunohistochemistry for immunoglobulin G (IgG), which are reliable markers of BBB breakdown in this model. We found that 24 h after the correction of hyponatremia, both urea and lixivaptan did not induce intraparenchymal extravasation of IgG as opposed to HS (Figure 3a–d), and these results were confirmed by EB analysis (Figure 3j). However, 6 days after the treatment of hyponatremia began, several large areas of broken BBB (as evidenced by intraparenchymal extravasation of IgG) were seen in animals treated with lixivaptan or HS compared with animals treated with urea (Figure 3e–i).

Rapid correction of hyponatremia with urea causes less microglial activation and astrocyte damage than treatment with lixivaptan or HS

We recently reported that astrocytes were lost in the brain of animals with ODS.[6] We wanted to know the effect of urea and lixivaptan on astrocyte viability. At 24 h after the beginning of the correction of SNa, animals treated with HS saline displayed larges areas with loss of the glial fibrillary acidic protein (GFAP) immunoreactivity. In contrast, animals treated with lixivaptan or urea had much less areas of astrocyte loss ($P<0.01$ for GFAP-depleted areas in HS vs. urea or lixivaptan). Six days after the correction of hyponatremia started, the brain of animals treated with the V2R antagonist and HS showed extensive areas of astrocyte death, whereas there was only minimal astrocyte loss in few of the animals treated with urea (Figure 4m, $P<0.01$ for lixivaptan or HS vs. urea). In animals treated with lixivaptan, astrocyte-depleted regions were surrounded by an intense astrogliosis, as previously reported[6,29] (Figure 4a–c and Supplementary Figure S4 online).

Microglial activation is another hallmark of ODS, and we therefore wanted to assess the ability of both treatments to decrease microglial activation. Similar to animals treated with HS, rats treated with lixivaptan displayed strong evidence of microglial activation in the hippocampus, basal ganglia, and cortical regions, whereas urea-corrected animals had no evidence of microglial activation (Figure 4i–l and n). We also established that in lixivaptan-treated animals astrocyte-depleted regions were the same regions subject to demyelination and microglial infiltration (Supplementary Figure S4 online).

Correction of hyponatremia with urea but not with lixivaptan can alleviate myelinolysis

We next addressed the presence and magnitude of demyelinative regions in animals of each group, and the brains of surviving animals were processed for immunohistochemistry with myelin antibody. All the six surviving rats treated with HS had demyelinative lesions (in experiment 2, the only surviving rat died during day 10 before killing and was not examined histologically to avoid autolysis artifact); in contrast, only 3 out of the 28 surviving animals treated with urea displayed minimal signs of demyelination, and 8 out of the 13 animals in the lixivaptan group showed demyelinative lesions in the brain.

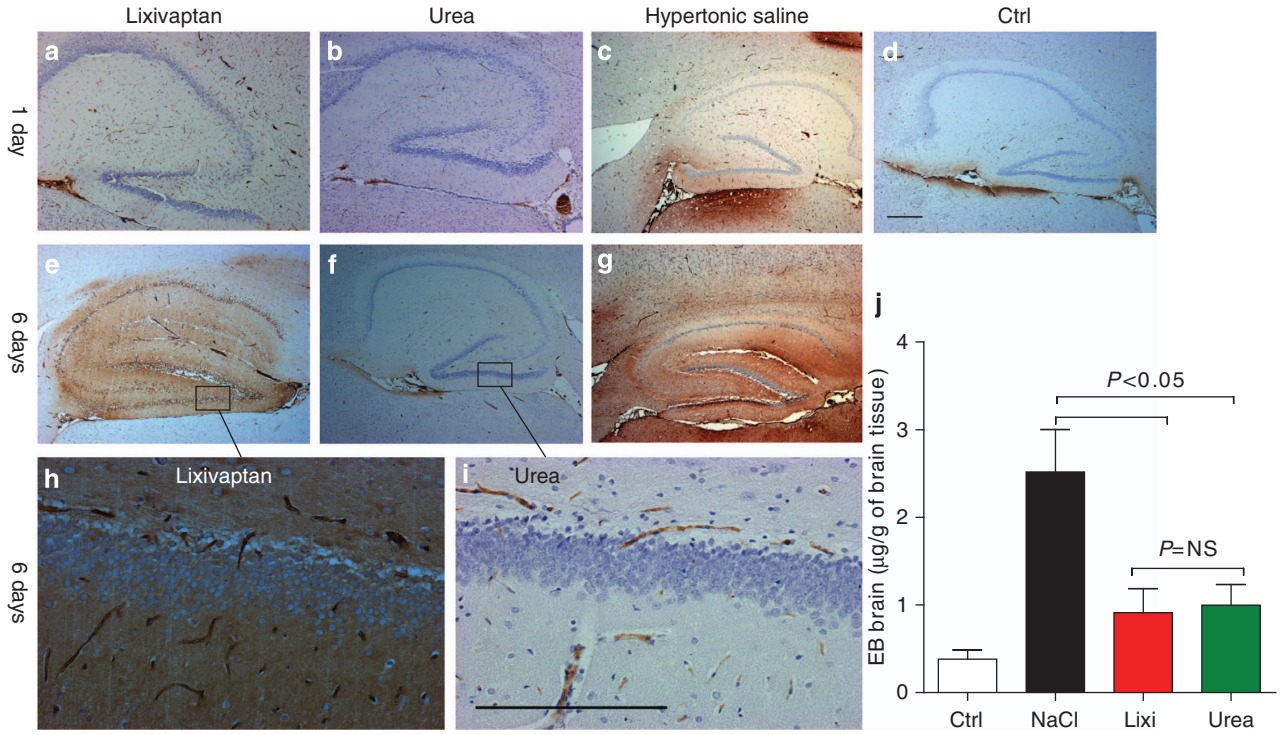

Figure 3 | Blood–brain barrier after hyponatremia correction with lixivaptan, hypertonic saline, or urea. (**a**, **b**) One day after the correction of hyponatremia with lixivaptan or urea only, minor intraparenchymal immunoglobulin G (IgG) is seen compared with (**d**) control. On the other hand, as shown in panel (**c**), extensive IgG staining is seen in animals treated with hypertonic saline. (**e–g**) At 6 days after the beginning of hyponatremia correction, significant intraparenchymal extravasation of IgG is seen in animals corrected with lixivaptan (**d** and higher magnification in **f**) and hypertonic saline (**g**). In animals treated with urea, only intravascular staining is present, suggesting no alteration in the BBB permeability (**f**) and higher magnification in panel (**i**). Graph (**j**) shows that there is no significant difference in the Evans blue (EB) content in animals treated with lixivaptan and in animals treated with urea 1 day after the correction began as opposed to animals treated with hypertonic saline ($n = 5$–8 for panel **h**, $P =$ NS for lixivaptan vs. urea or control and $P < 0.05$ for NaCl vs. urea or lixivaptan by analysis of variance test). Bar = 200 µm.

DISCUSSION

The treatment of severe hyponatremia remains a challenge, as it should put into balance the risk of neurological damage associated with too rapid correction of SNa and the risk of hyponatremic encephalopathy from persistent severe hyponatremia.[30] Available choices include HS, urea, and the vasopressin receptor antagonists.[31] The use of these three agents could be associated with unpredictable rise in SNa during correction of hyponatremia. Overly rapid increase in SNa by HS has been associated with ODS,[3] but little is known about the risk of ODS after treatment with vaptans in the event of a too sharp increase of the SNa. On the other hand, our previous experimental studies have shown that urea might be protective during rapid correction of severe hyponatremia.[20,21] Here, using two different experimental paradigms, which included a large series of animals, we have demonstrated that despite a comparable rise in serum sodium animals treated with urea had a better outcome than animals treated with lixivaptan or HS.

The increased mortality associated with overcorrection of hyponatremia with lixivaptan that we observed is in line with previous preliminary work published as abstracts using a different V2R antagonist.[11,12]

Rapid correction of hyponatremia with urea compared with either lixivaptan or HS significantly decreased brain microglial activation, significantly reduced changes in the BBB permeability, increased astrocyte viability, and reduced brain histological evidence of demyelination 6 days after the beginning of the correction of hyponatremia.

The clinical importance of our result is unclear at this stage. Although we observed significant mortality in animals treated with vaptans, the clinical experience with that drug class does not confirm an increased risk of osmotic demyelination, as so far only one case of ODS with the use of vaptans in monotherapy has been reported.[13] In addition, the increments of serum sodium achieved in this experimental study are seldom seen in clinical practices, and it is likely that the vaptans when used to correct hyponatremia within the prescribed guidelines are not associated with a significant risk of ODS.

As of the protection afforded by urea, some experimental caveats need to be taken into account. First of all, because of its short half-life, we administered urea every 6 h. Although repeated handling and injections were previously shown not to affect the outcome in experimental ODS,[21] there is a theoretical possibility that different injection schedules could

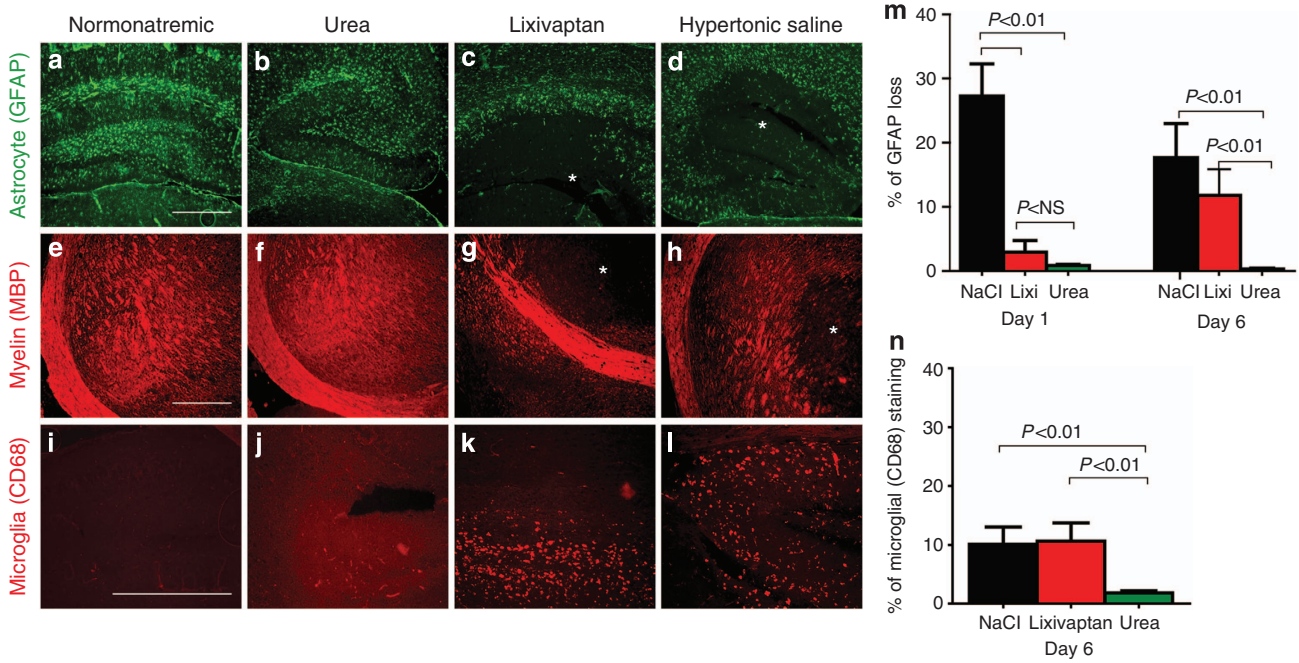

Figure 4 | Histological manifestations in the brain of animals corrected with lixivaptan and urea 6 days after the beginning of hyponatremia correction. (**a**–**d**) Astrocyte staining (glial fibrillary acidic protein (GFAP)) in all the three groups. Normal astrocyte staining is seen in the hippocampus of control animals (**a**) and animals treated with urea (**b**), whereas animals treated with lixivaptan (**c**) and hypertonic saline (**d**) showed significant astrocyte loss (*). (**e**–**h**) Myelin staining in the basal ganglia. Normal and homogeneous myelin staining is seen in control animals and in animals corrected with urea (**d** and **e**, respectively). On the contrary, significant myelin loss (*) is seen in animals corrected with (**g**) lixivaptan or (**h**) hypertonic saline. (**i**–**l**) Microglial staining (CD68) in the hippocampus. No microglial staining was seen in controls and animals corrected with urea. In animals corrected with lixivaptan or hypertonic saline, significant microglial staining was observed, (**k**) and (**l**). Bar = 500 μm. Graph (**m**) shows that there is no significant difference between astroglial (GFAP) staining in animals treated with lixivaptan and urea 1 day after the correction of hyponatremia began, whereas animals treated with hypertonic saline have several large astrocyte-depleted brain regions already. However, 6 days after the beginning of correction of hyponatremia, animals treated with lixivaptan or hypertonic saline had bigger areas of GFAP loss than those corrected with urea (P = NS by unpaired t-test for GFAP staining 1 day after the correction, $P < 0.01$ for GFAP 6 days after the correction, $n = 6$–11). Graph (**n**) shows that animals treated with lixivaptan or hypertonic saline had a significantly higher area of microglial infiltration than animals treated with urea ($P < 0.01$ for microglial infiltrated area 6 days after the beginning of sodium correction).

have affected the results. In addition, the different kinetics of serum sodium increase in the first hours might have affected the findings, as we measured serum sodium only at 12 h. In this regard, it is worthly to note that a recent report has suggested that with the use of tolvaptan the increment of serum sodium in the first 3 h could be a determinant for future demyelination.[32] Also, this study did not determine which of the presence of high blood urea during the correction of hyponatremia or the osmotic diuresis induced by urea is the protective factor. However, some insight on the matter could be derived from the fact that animals with acute renal failure were also shown to be resistant to ODS.[33]

Notwithstanding those technical limitations, there are some new insights into the physiopathology of ODS that could be derived from our results. These experiments shed new light into the role of the BBB permeability during ODS. We observed that despite a very high increase of sodium obtained by administering urea or lixivaptan 24 h after the correction, no changes in the permeability of the BBB were seen. On the contrary, a similar increment of SNa obtained with a bolus injection of HS resulted in a diffuse opening of the BBB at 24 h. HS induces a brisk increase in SNa as opposed to urea or V2R antagonist; from that standpoint, these results first confirm that the early opening of the BBB (at 24 h) is not a major determinant in ODS morbidity, as animals treated with lixivaptan still fared poorly despite a closed BBB 24 h after the treatment. Second, our results suggest that a sharp and high increase in the SNa is needed to induce early (24 h) changes in the permeability of the BBB.

This supports the hypothesis that the opening of the BBB in ODS might be a two-step process where the first step is secondary to abrupt osmotic stress with astrocyte death and the second step is secondary to the inflammation that goes along with microglial activation and demyelination.

The absence of astrocyte loss in animals treated with lixivaptan 24 h after the beginning of the correction, although astrocyte loss was evident 5 days later, also hints that astrocyte demise might also depend on both the magnitude and the intensity of the osmotic stress sustained by these cells.

After rapid correction of hyponatremia with HS, brain sodium value increases above the normal level (in normonatremic animals); this was called an overshoot in the brain sodium.[4,34] This peculiar finding is thought to induce brain cell hyperionization, which could be deleterious to the cell metabolism. Urea has been reported to prevent brain sodium overshooting, and the ability of urea to protect the brain during rapid correction of hyponatremia might rely on these properties.[20] It is also worth mentioning that although urea has been widely seen as a protein denaturant it has been shown to protect from freezing stress and oxidative stress in some settings.[16–18] Interestingly, hypertonicity from NaCl induced DNA damage as opposed to urea-induced hypertonicity in kidney cells,[19] and accordingly our preliminary studies have shown that rapid correction of hyponatremia with urea as opposed to HS induces a different pattern of gene expression in the brain, which may suggest that there are indeed some specific effects of urea on the brain during correction of severe hyponatremia.[35]

In summary, we studied the effects of exogenous urea or a vasopressin receptor antagonist (lixivaptan) vs. HS in a rat model of hyponatremia and we found that overcorrection of hyponatremia with lixivaptan and HS induces severe neurological manifestations and demyelination in the murine brain, whereas on the other hand overcorrection of hyponatremia with urea resulted in significantly lower mortality and neurological impairment.

It is important to mention that these studies were set in extreme conditions of SNa correction that hardly reflect the daily clinical practice. Indeed, the risk of ODS after hyponatremia is corrected within the recommended range regardless of the drug used is extremely low. Further studies are needed to fully elucidate the mechanisms associated with the protective effect of urea on ODS.

MATERIALS AND METHODS

Animals, induction, and correction of hyponatremia

For all the experiments, male Wilstar rats, aged 8–10 weeks and weighing 200–350 g, were used. They were allowed to adapt in individual cages for a week with *ad libidum* access to water and food. Procedures were performed in accordance with guidelines for animal care at Université Libre de Bruxelles.

Hyponatremia was induced as described in earlier work[22,36] by a combination of osmotic minipumps (Model 2001 Alzet, Palo Alto, CA) filled with 4 μg/ml desmopressine acetate (Ferring, Malmoe, Sweden) and a liquid formulated diet for 4 days.

Hyponatremia was corrected according to the experimental paradigms and group allocation, as detailed below (Supplementary Figure S1 online).

Reagents. Urea solution was made fresh, by dissolving 24 g of urea in 100 ml of D5W, and the mixture was allowed to reach room temperature. Lixivaptan solution was made by dissolving 50 mg of lixivaptan powder (BOCscience, Shirley, NY) in 50 ml of D5W at a concentration of 1 mg/ml.

Experiment 1. In this first experimental paradigm, hyponatremic animals were divided into three groups: Group 1 (Urea, $n = 22$) had SNa corrected with urea solution administered intraperitoneally over 5 min at the dose of 0.8 ml/100 g every 6 h for a total of four doses.

Group 2 (Lixivaptan, $n = 23$) received a single intraperitoneal (ip) injection of lixivaptan solution (2 ml/100 g).

Group 3 (HS, $n = 25$) received a single ip injection of 1.5 M NaCl at a concentration of 1.5 ml/100 g of body weight. In this experiment, SNa was measured before the correction and 24 h after the beginning of correction for all animals.

To investigate the kinetics of SNa increase with this paradigm, in preliminary studies, 5–10 animals of each group had SNa measured 12 h after the initiation of SNa correction.

Experiment 2. The second set of experiments was designed to determine whether the rate at which the SNa increment is achieved affects the outcome. Animals were divided into three groups:

Group 4 (Urea, $n = 16$) consisted of hyponatremic animals corrected with ip urea, as in experiment 1.

Group 5 (Lixivaptan, $n = 17$) consisted of hyponatremic animals corrected with lixivaptan, which was administered in two divided ip doses in order to get an SNa increment at 12 h equal to around half of the total SNa increment. The first dose of lixivaptan was 0.5 mg/100 g of body weight, followed by 1.5 mg/100 g of body weight 12 h later.

Group 6 (HS, $n = 15$) consisted of animals that received two divided ip doses of 1.5 M HS at a concentration of 0.75 ml/100 g for the first dose and at a concentration of 1.25 ml/100 g for the second dose, which was given 12 h after the first dose.

In the second experiment, SNa was measured 12 and 24 h after the beginning of correction for all animals. At 24 h after the beginning of correction, rats were switched again to normal pellet diet and water. Blood samples (0, 3 ml) were collected via tail transection at specified times according to the group allocation, and electrolyte measurements were taken using MODULAR p800, Roche (Roche Diagnostics, Vilvoorde, Belgium).

Evaluation of neurological manifestations and mortality

Rats were weighed and observed daily for occurrence of neurological manifestations graded as follows[37]: 6 = No neurological manifestations; 5 = slow or awkward gait; 4 = limb weakness and/or paralysis; 3 = seizures; 2 = severe motor deficits; 1 = complete inability to move; and 0 = death. All animals were allowed to survive until 6 days after the beginning of the correction of SNa.

Immunohistochemistry and immunofluorescence

Brain processing for histology and immunohistochemistry/immunofluorescence including tyramide amplification procedure was performed as previously detailed[36] 24 h and 6 days after the beginning of hyponatremia correction in each group of animals. Antibodies used included anti-MBP (clone SMI 32 at 1/1000 Abcam, Cambridge, England), anti-GFAP (ab 5260 from Abcam and clone GA5 from Imgenex, San Diego, CA), anti-CD68 (clone ED1 at 1/200—AbDserotec, Oxford, England), and goat anti-rat IgG (1/50 Vector Labs, Brussels, Belgium). Fluorescent reagents used were goat anti-mouse Alexa 594 (Invitrogen, Merelbeke, Belgium) and tyramide fluorescein isothiocyanate (PerkinElmer, Zaventem, Belgium).

Image quantification

For astroglial (GFAP) and microglial (CD68) quantification, the whole-stained slides were captured at up to ×20 magnification with an automated microscope (Hamamatsu Nanozoomer C96000-01,

Hamamatsu Photonics, Belgium). The image was imported to ImageJ software (US NIH, Bethesda, MD), and individual images were then converted to binary image. ImageJ threshold function was then used with rigorous microscopic control to delineate only areas of GFAP signal loss or CD68 microglial-positive staining. Those areas were then quantified and divided by the area of the entire brain image to obtain the proportion of brain surface with astrocyte loss or microglial infiltration.

Evaluation of BBB by EB dye extravasation

EB was used to determine the BBB permeability 24 h after the correction of the hyponatremia in animals treated with lixivaptan, HS, and urea. For EB analysis, hyponatremia induction and correction was performed similarly to experiment 1. However, animals were used 24 h after the correction for BBB permeability analysis with EB. EB dye extraction was done using formamide, as previously described.[6]

Statistical analysis

Results were expressed as mean ± s.e.m. All variables were assayed for normality by Shapiro–Wilk test. Paired and unpaired *t*-test, analysis of variance one- or two-way tests, and Kruskal–Wallis tests were used as appropriate. Fischer's exact test on proportions and Kaplan–Meir survival analysis with log-rank test were also used to compare mortality in the different groups. All the statistical analyses were performed using the StatDirect software (Cambridge, England), and graphs were designed using the GraphPad software (La Jolla, CA). P values <0.05 were considered to be significant.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. Schematic representation of the experimental design.
Figure S2. Increment of serum sodium after a single bolus of NaCl or lixivaptan compared with 4 divided doses (q6 hrs) of urea.
Figure S3. Overall mortality in all the six groups of animals studied.
Figure S4. Colocalization of astrocyte, myelin, and microglia in the brain of animals treated with lixivaptan 5 days after the correction of hyponatremia.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

REFERENCES

1. Sterns RH, Cappuccio JD, Silver SM, Cohen EP. Neurologic sequelae after treatment of severe hyponatremia: a multicenter perspective. *J Am Soc Nephrol.* 1994; **4**: 1522–1530.
2. Sterns RH, Riggs JE, Schochet SS Jr. Osmotic demyelination syndrome following correction of hyponatremia. *N Engl J Med* 1986; **314**: 1535–1542.
3. Mohmand HK, Issa D, Ahmad Z *et al.* Hypertonic saline for hyponatremia: risk of inadvertent overcorrection. *Clin J Am Soc Nephrol* 2007; **2**: 1110–1117.
4. Verbalis JG, Gullans SR. Rapid correction of hyponatremia produces differential effects on brain osmolyte and electrolyte reaccumulation in rats. *Brain Res* 1993; **606**: 19–27.
5. Lien YH, Shapiro JI, Chan L. Study of brain electrolytes and organic osmolytes during correction of chronic hyponatremia. Implications for the pathogenesis of central pontine myelinolysis. *J Clin Invest* 1991; **88**: 303–309.
6. Gankam Kengne F, Nicaise C, Soupart A *et al.* Astrocytes are an early target in osmotic demyelination syndrome. *J Am Soc Nephrol* 2011; **22**: 1834–1845.
7. Decaux G, Soupart A, Vassart G. Non-peptide arginine-vasopressin antagonists: the vaptans. *Lancet* 2008; **371**: 1624–1632.
8. Cho C, Logan JL, Lien Y-HH. Massive aquaresis after tolvaptan administration and albumin infusion in a patient with alcoholic cirrhosis. *Am J Med* 2012; **125**: e5–e6.
9. Schrier RW, Gross P, Gheorghiade M *et al.* Tolvaptan, a selective oral vasopressin v2-receptor antagonist, for hyponatremia. *N Engl J Med* 2006; **355**: 2099–2112.
10. Jaber BL, Almarzouqi L, Borgi L *et al.* Short-term efficacy and safety of vasopressin receptor antagonists for treatment of hyponatremia. *Am J Med* 2011; **124**: 977.e971–977.e979.
11. Takagi H, Sugimura Y, Suzuki H *et al.* Overly rapid correction of severe hyponatremia with vasopressin receptor antagonists poses a risk of inducing osmotic demyelination syndrome. *Endocr Abstracts* 2012; **29**: 1144.
12. Soupart A, Silver S, Schroeder B *et al.* Comparison of mortality and brain composition in hyponatremic rats treated with vassopressin V1-V2 receptor antagonist and water diuresis (Abstract). *J Am Soc Nephrol* 2003; **14**: 747A.
13. Malhotra I, Gopinath S, Janga KC *et al.* Unpredictable nature of tolvaptan in treatment of hypervolemic hyponatremia: case review on role of vaptans. *Case Rep Endocrinol* 2014; **2014**: 4.
14. Decaux G, Unger J, Brimioulle S *et al.* Hyponatremia in the syndrome of inappropriate secretion of antidiuretic hormone. Rapid correction with urea, sodium chloride, and water restriction therapy. *JAMA* 1982; **247**: 471–474.
15. Soupart A, Coffernils M, Couturier B *et al.* Efficacy and tolerance of urea compared with vaptans for long-term treatment of patients with SIADH. *Clin J Am Soc Nephrol* 2012; **7**(5): 742–747.
16. Costanzo JP, Lee RE Jr. Cryoprotection by urea in a terrestrially hibernating frog. *J Exp Biol* 2005; **208**: 4079–4089.
17. Costanzo JP, Lee RE Jr. Urea loading enhances freezing survival and postfreeze recovery in a terrestrially hibernating frog. *J Exp Biol* 2008; **211**: 2969–2975.
18. Wang X, Wu L, Aouffen M *et al.* Novel cardiac protective effects of urea: from shark to rat. *Br J Pharmacol* 1999; **128**: 1477–1484.
19. Kültz D, Chakravarty D. Hyperosmolality in the form of elevated NaCl but not urea causes DNA damage in murine kidney cells. *Proc Natl Acad Sci* 2001; **98**: 1999–2004.
20. Soupart A, Schroeder B, Decaux G. Treatment of hyponatraemia by urea decreases risks of brain complications in rats. Brain osmolyte contents analysis. *Nephrol Dial Transplant* 2007; **22**: 1856–1863.
21. Soupart A, Stenuit A, Perier O *et al.* Limits of brain tolerance to daily increments in serum sodium in chronically hyponatraemic rats treated with hypertonic saline or urea: advantages of urea. *Clin Sci (Lond)* 1991; **80**: 77–84.
22. Verbalis JG, Martinez AJ. Neurological and neuropathological sequelae of correction of chronic hyponatremia. *Kidney Int* 1991; **39**: 1274–1282.
23. Sterns RH. Severe symptomatic hyponatremia: treatment and outcome. A study of 64 cases. *Ann Intern Med* 1987; **107**: 656–664.
24. Laureno R. Rapid correction of hyponatremia: cause of pontine myelinolysis? *Am J Med* 1981; **71**: 846–847.
25. Laureno R. Central pontine myelinolysis following rapid correction of hyponatremia. *Ann Neurol* 1983; **13**: 232–242.
26. Verbalis JG, Martinez AJ. Determinants of brain myelinolysis following correction of chronic hyponatremia in rats. *Vasopressin* 1991; **208**: 539–547.
27. Costello-Boerrigter L, Boerrigter G, Burnett J Jr. Pharmacology of vasopressin antagonists. *Heart Fail Rev* 2009; **14**: 75–82.
28. Adler S, Verbalis JG, Williams D. Effect of rapid correction of hyponatremia on the blood-brain barrier of rats. *Brain Res* 1995; **679**: 135–143.
29. Iwama S, Sugimura Y, Suzuki H *et al.* Time-dependent changes in proinflammatory and neurotrophic responses of microglia and astrocytes in a rat model of osmotic demyelination syndrome. *GLIA* 2011; **59**: 452–462.
30. Berl T. Treating hyponatremia: damned if we do and damned if we don't. *Kidney Int* 1990; **37**: 1006–1018.
31. Sterns RH, Nigwekar SU, Hix JK. The treatment of hyponatremia. *Semin Nephrol* 2009; **29**: 282–299.
32. Takagi H, Sugimura Y, Suzuki H *et al.* Minocycline prevents osmotic demyelination associated with aquaresis. *Kidney Int* 2014; e-pub ahead of print 23 April 2014 (doi:10.1038/ki.2014.119).

33. Soupart A, Penninckx R, Stenuit A, Decaux G. Azotemia (48 h) decreases the risk of brain damage in rats after correction of chronic hyponatremia. *Brain Res* 2000; **852**: 167–172.
34. Soupart A, Silver S, Schrooeder B *et al.* Rapid (24-hour) reaccumulation of brain organic osmolytes (particularly myo-inositol) in azotemic rats after correction of chronic hyponatremia. *J Am Soc Nephrol* 2002; **13**: 1433–1441.
35. Gankam Kengne F, Couturier B, Soupart A *et al.* Correction of hyponatremia with saline versus urea induces differential gene expression changes in the brain (Abstract). *J Am Soc Nephrol* 2012; **23**: 1.
36. Gankam Kengne F, Soupart A, Pochet R *et al.* Re-induction of hyponatremia after rapid overcorrection of hyponatremia reduces mortality in rats. *Kidney Int* 2009; **76**: 614–621.
37. Sugimura Y, Murase T, Takefuji S *et al.* Protective effect of dexamethasone on osmotic-induced demyelination in rats. *Exp Neurol* 2005; **192**: 178–183.