

Original Articles

## Carbamazepine can induce kidney water absorption by increasing aquaporin 2 expression

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### Abstract

**Background.** Carbamazepine (Carba) is an anticonvulsant and psychotropic drug used widely for the treatment of intellectual disability and severe pains, but the incidence of hyponatremia is a common related occurrence. This hyponatremia is frequently attributed to a SIADH induced by this drug. It is also known that Carba is used to decrease the urinary volume in Diabetes Insipidus (DI) because it has an antidiuretic effect. Lithium (Li) is one of the most important drugs used to treat bipolar mood disorders. However Li has the undesirable capacity to induce DI. Nowadays, the association of these drugs is used in the treatment of patients with psychiatric and neurological problems.

**Methods.** *In vivo* and *in vitro* (microperfusion) experiments were developed to investigate the effect of Carba in the rat Inner Medullary Collecting Duct (IMCD).

**Results.** The results revealed that Carba was able to stimulate the V2 vasopressin receptor-Protein G complex increasing the water permeability (Pf) and water absorption. *In vivo* studies showed that in rats with lithium-induced DI, Carba decreased the urinary volume and increased the urinary osmolality. AQP2 expression was increased both in normal IMCD incubated with Carba and in IMCD from lithium-induced DI after Carba addition to the diet, when compared with the control.

**Conclusion.** These results showed that the hyponatremia observed in patients using this anticonvulsant drug, at least in part, is due to the Carba capacity to increase IMCD's Pf and that the Lithium-Carbamazepine association is beneficial to the patient.

**Keywords:** aquaporin 2; carbamazepine; diabetes insipidus; lithium; water absorption

### Introduction

Carbamazepine is an anticonvulsant and psychotropic medication commonly used in the treatment of patients with epilepsy or intellectual disability. This drug has been also used to decrease the urinary volume in diabetes insipidus because of its antidiuretic effect [1,2]. Nevertheless,

the incidence of the hyponatraemia in neurological patients using carbamazepine is a common occurrence [3–5]. This carbamazepine antidiuretic effect is not well characterized, and frequently, it has been suggested to be responsible for the stimulation of the vasopressin release from the pituitary gland (syndrome of inappropriate antidiuretic hormone secretion, SIADH). However, there has been evidence of a possible effect directly on the renal tubule. Lithium carbonate is one of the most important drugs used in treating bipolar mood disorders [6], and it is also used as a neuroprotective drug for the treatment of neurodegenerative diseases such as amyotrophic lateral sclerosis and Alzheimer's disease. Nonetheless, lithium carbonate treatment is often associated with nephrogenic diabetes insipidus (NDI) with polyuria, polydipsia and a reduced ability to concentrate urine [6]. Chronic lithium carbonate treatment causes a substantial decrease in aquaporin 2 (AQP2) expression in rat medullary collecting ducts, and lithium-induced NDI may be due, at least partly, to a reduction in AQP2 expression [6,7]. AQP2 is a water channel located in the principal cells of renal collecting duct in the apical plasma membrane and intracellular vesicles. Vasopressin increases the water permeability stimulating the insertion of AQP2 into the plasma membrane, and mutations in this water channel can cause severe NDI [7]. Nowadays, the association of carbamazepine (Carba) and lithium carbonate (Li) is used in the treatment of patients with psychiatric and neurological disorders.

Thus, this work was designed to investigate the effect of carbamazepine in the inner medullary collecting duct (IMCD) and its effect in the lithium chloride-induced NDI in Wistar rats.

### Materials and methods

Male Wistar rats, weighing 170–180 g, were obtained from the animal facilities of University of São Paulo Medicine School. They were maintained under standard laboratory conditions and fed a normal pellet diet and tap water *ad libitum*.

#### *In vitro* studies

**Microperfusion.** Isolated IMCDs were perfused by previously described techniques [8,9] in the absence of ADH and in the presence of Carba, in

order to determine the osmotic water permeability (Pf). In the next groups of experiments, the water permeability was determined in the presence of Carba+AV2 (AV2—antagonist of V2 receptor-G complex in basolateral membrane) and Carba+H8 (H8—inhibitor of PKA). Tubules were isolated from a small kidney slice that was immersed in a dish of chilled Ringer-HCO<sub>3</sub> buffer, oxygenated and kept at pH 7.4 by bubbling the solution with 5% CO<sub>2</sub>–95% O<sub>2</sub>. After isolation, the segment was transferred to a temperature-regulated chamber (37°C) mounted on the stage of an inverted microscope.

The perfusion solution used was a 295±5 mOsmol/kgH<sub>2</sub>O Ringer-HCO<sub>3</sub>, and the bath solution was made hypertonic (510±5 mOsmol/kgH<sub>2</sub>O) by the addition of NaCl. FD&C green dye was added to the perfusate as a visual marker. Net water absorption ( $J_w$ ) was measured with [<sup>14</sup>C]inulin dialysed immediately before the experiments. The baths were checked for osmolality and pH, with an osmometer (Advanced Instruments) and with a pHmeter (Iris 7; Tecnow, São Paulo, Brazil), respectively. The bath fluid was changed every 10 mins to reduce the effect of evaporation and consequently to avoid an increase in the osmotic gradient. The isotopic concentration was determined by a liquid scintillator spectrometer (Tri-Carb 1600TR; Packard, Downers Grove, IL, USA). The data for each period are the average of 3–4 collections.

**Tubule suspension.** The tubule strips were dissected from the renal medullas from normal rats in the same cold solution used in the micro-perfusion experiments. These strips were incubated with 10<sup>-5</sup> M carbamazepine at 37°C, and the solution was bubbled with 5% CO<sub>2</sub> and 95% O<sub>2</sub> for 30 mins to determine the expression of AQP2. The control medullas were incubated in the same fashion without Carba [10].

#### *In vivo studies*

During the 3-week study period, all animals were fed standard rat chow with a fixed concentration of sodium chloride (0.5%) and given *ad libitum* access to tap water. Experimental nephrogenic diabetes insipidus was induced by administration of lithium chloride (Li) [6], and the following protocol was carried out: the rats were divided into four groups: (i) control (fed standard rat chow for 3 weeks,  $n=5$ ); (ii) Li (fed standard rat chow + Li 40 mM/kg/food for 3 weeks  $n=5$ ); (iii) Li+Carb (fed standard rat chow + Li 40 mM/kg/food for 3 weeks + Carba 400 mg/kg per body weight (bw) for the last 2 weeks of that period,  $n=4$ ); and (iv) Carba (fed standard rat chow + Carba 250 mg/kg/bw for 3 weeks,  $n=5$ ).

Baseline urine samples were collected over a period of 24 h by placing the animals in individual cages (Day 0). On Day 1, the rats were subdivided into the four groups mentioned above. At the end of Week 1 and again at the end of Week 3, the animals were placed in metabolic cages to collect 24-h urine samples. At the end of Week 3, rat weights were measured, and the animals were killed; blood was collected for measurements of sodium (Na<sup>+</sup>) and potassium (K<sup>+</sup>) plasma levels. At the same time, the renal medulla was separated to determine the expression of AQP2.

**Western blot: preparation of membrane fractions.** Medulla samples were homogenized in cold isolation solution (20 mM Mannitol, 80 mM Hepes and 41 mM KOH; pH 7.5) containing protease inhibitors (cocktail protease inhibitor, Sigma Chemical, St. Louis, MO, USA) using a Teflon pestle glass homogenizer (Schmidt and Co, Frankfurt/M, Germany) [11]. The homogenates were centrifuged at low speed (4000 g) for 15 mins at 4°C in order to remove the nuclei and cell debris. Subsequently, the supernatants were centrifuged at 200 000 g for 1 h at 4°C (rotor 50Ti; Beckman Instruments, Palo Alto, CA, USA) to produce a pellet containing membrane fractions enriched with plasma membranes and intracellular vesicles. Protein concentration was determined for each sample using the Bradford method (Bio-Rad Laboratories, Richmond, CA, USA).

**Electrophoresis and immunoblotting.** The proteins were separated on denaturing 12% SDS–polyacrylamide gels by electrophoresis [11]. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane by wet electroblotting for 90 mins. Blots were blocked for 60 mins at 4°C with 5% non-fat dry milk in PBS-T, pH 7.5 (phosphate-buffered saline; in millimolar: 100 NaCl, 80 Na<sub>2</sub>HPO<sub>4</sub>, 20 NaH<sub>2</sub>PO<sub>4</sub> and 0.1% Tween-20). Blots were incubated with the AQP2 antibody (1:10 000 dilution) and with the control actin antibody (1:2000) overnight, and then washed and incubated with the second antibody (anti-goat secondary antibody HRP-conjugated, diluted at 1:10 000) for 1 h. Subsequent de-

tection of the specific proteins was carried out by enhanced chemiluminescence (ECL, Amersham), according to the manufacturer's instructions. Pre-stained protein markers (Sigma Chemical Co) were used for molecular weight determinations.

**Quantification of AQP2 kidney levels.** Enhanced chemiluminescence films presenting bands within the linear range were scanned using the Image Master VDS (USA). For AQP2, both the 29- and 35–50-kDa bands (corresponding to two different states of glycosylation) were quantified by densitometric analysis. Densitometry results are reported as integrated values (area × density of the band) and expressed in percentage when compared to control actin protein abundance (100%).

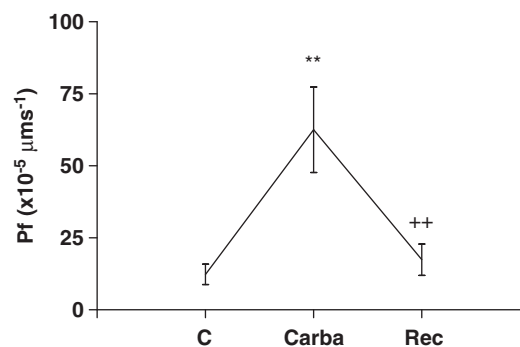
**Statistics.** Differences among the means of multiple parameters were analysed by ANOVA followed by the Student–Newman–Keuls test. Differences between two parameters were analysed by either paired *t*-test or by non-parametric methods (Mann–Whitney test). Values of  $P<0.05$  were considered significant.

**Chemicals.** The isotopic materials used were from Amersham International and New England Nuclear. Carbamazepine was kindly supplied by the Aché/Biosintética Laboratory, SP, Brazil. The other drugs were purchased from Sigma Chemical Saint Louis, MO, USA; Santa Cruz Biotechnology, Santa Cruz, CA, USA; Amersham Biosciences, Sweden; and Pharmacia Biotech, Uppsala, Sweden. The V2 receptor antagonist was kindly supplied by Dra Claudine Serradeil-Le Gal from Sanofi-Recherche.

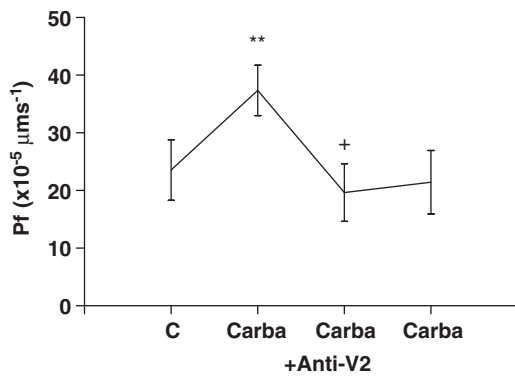
## Results

### *In vitro study*

**Microperfusion.** The osmotic water permeability (Pf × μm/s) was measured in the final third of the IMCD (IMCD<sub>3</sub>) directly dissected from normal rat medullas. The carbamazepine (10<sup>-5</sup> M) was added to the bath solution in the absence of ADH. Figure 1 shows that, in the presence of carbamazepine ( $n=6$ ), the water permeability increased from 12.3±3.6 (control) to 62.6±14.8 (Carba) ( $P<0.01$ ) and recovered to 17.4±5.5 (Rec) ( $P<0.01$ ). Figure 2 shows that the addition of 10<sup>-6</sup> M of the V2 antagonist (AV2-SR-121463) in the bath plus 10<sup>-5</sup> M of Carba (Carba+AV2) ( $n=6$ ) decreased the water permeability from 37.4±4.4 (Carba) ( $P<0.01$ ) to 19.6±5.0 (Carba+AV2) ( $P<0.05$ ). In Figure 3, no significant difference was observed, showing that the V2 receptor in the basolateral membrane is activated by the action of carbamazepine ( $n=4$ ). Figure 4 shows that the addition of 10<sup>-6</sup> M of H8 (*N*-[2-(methylamino)ethyl]-5-isoquinoline-



**Fig. 1.** Carbamazepine effect on water permeability from normal rats IMCD ( $n=6$ ); \*\* $P<0.01$  vs. control (C) and ++ $P<0.01$  vs. Carba.

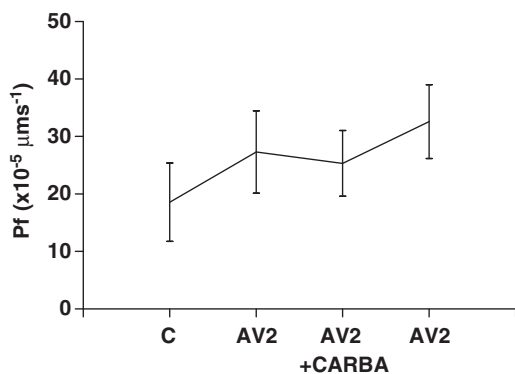


**Fig. 2.** Carbamazepine+Anti-V2 effect on water permeability from normal rats IMCD ( $n=6$ ); \*\* $P<0.01$  vs. control (C); and + $P<0.05$  vs. Carba.

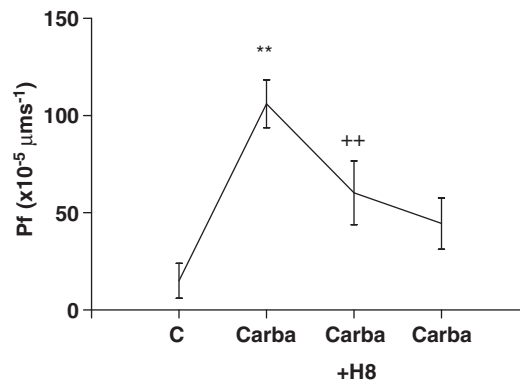
sulphonamide hydrochloride) in the bath plus  $10^{-5}$  M of Carba (Carba+H8) also decreased the osmotic permeability from  $106.1 \pm 12.3$  (Carba) ( $P<0.01$ ) to  $60.3 \pm 16.4$  (Carba+H8) ( $P<0.01$ ), corroborating the results from Carba in the V2 receptor in the renal basolateral membrane.

**Tubule suspension.** Incubation of tubule suspension with  $10^{-5}$  M of Carba showed that AQP2 bands were more intense when compared with the control group. The control group mean was  $100.0 \pm 8.3$  ( $n=4$ ), and the Carba group mean was  $138.8 \pm 12.12$  ( $n=3$ ) (Figure 5).

**In vivo study.** All data are presented in Table 1. Lithium chloride concentration of 40 mmol/kg/diet induced NDI in animals that received treatment for 3 weeks, as shown by urine volume and water intake. Carbamazepine produced a recovery of these polyuria and polydipsia when administered together with the lithium chloride. The urinary osmolality was lower in all groups compared with the control group, and carbamazepine increased urinary osmolality when compared with the lithium chloride groups and with lithium chloride plus carbamazepine. The Carba group ( $n=5$ ) showed increased water ingestion and urinary volume, and decreased urinary osmolality compared with the control group. The animals that received lithium chloride ( $n=4$ ) showed weight loss, and the lithium chloride plus carbamazepine group ( $n=5$ ) continued with low weight. The con-



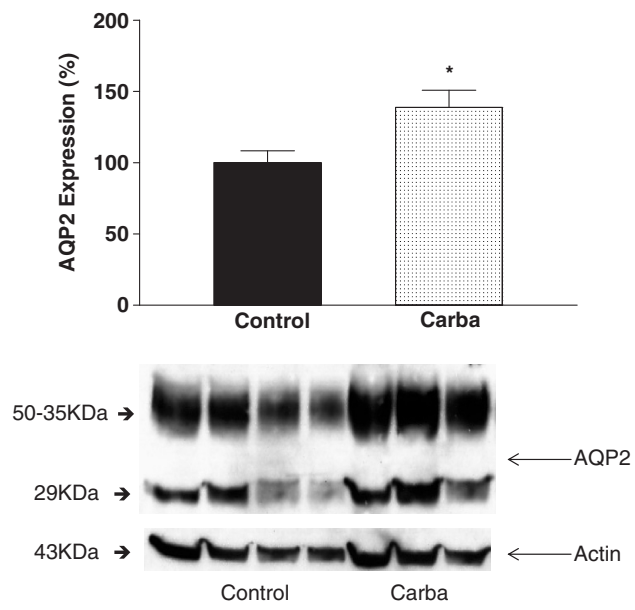
**Fig. 3.** Anti-V2+Carbamazepine effect on water permeability from normal rats IMCD ( $n=4$ ).



**Fig. 4.** Carbamazepine+H8 effect on water permeability from normal rats IMCD ( $n=4$ ); \*\* $P<0.01$  vs. control (C); and ++ $P<0.01$  vs. Carba.

rol animals and those which received carbamazepine ( $n=5$ ) showed a normal weight gain at the end of the experiment. Plasma creatinine was similar in all the groups. Creatinine clearance and sodium and potassium fractional excretion did not change in comparison with the control rats. Lithium plasma level was not detected in control group and in carbamazepine group, and was only detected in the lithium chloride and lithium chloride plus carbamazepine groups, but no statistical difference was found between these two groups. The plasma sodium and potassium levels did not change in any of the groups.

Western blotting analysis of AQP2 expression (Figure 6) showed that the animals that received lithium chloride presented water channel protein expression reduced  $\sim 50\%$  when compared with the control group. The carbamazepine addition to lithium chloride group was able to reverse the decrease in AQP2 expression  $\sim 20\%$ . Treatment with only carbamazepine did not alter AQP2 expression when compared with the control group and with the lithium



**Fig. 5.** Western blotting analysis from tubule suspension. Bands 29 and 35–50 kDa; \* $P<0.05$  vs. control.

**Table 1.** Biochemical results of the groups studied

Characteristic	Control (n=5)	Lithium (Li) (n=4)	Li+Carba (n=5)	Carba (n=5)
Weight of rats (g)	306.4±14.12	179.0±11.98*	132.5±7.77*	270.8±19.04****, *****
Urinary volume (mL/day)	10.68±3.0	62.60±6.0*	28.50±4.9***, ****	23.31±3.0***, ****
Ingestion of water (mL/day)	9.4±3.37	66.0±7.65*	47.75±2.29*, ****	35.0±3.88**, ****
U <sub>Osm</sub> (mOsm/Kg/H <sub>2</sub> )	819.6±175.7	149.4±18.05**	251.5±39.74**, *****	396.2±75.46**
F <sub>e</sub> Na (%)	0.15±0.01	0.10±0.03	0.12±0.02	0.11±0.02
F <sub>e</sub> K (%)	7.80±0.79	8.29±0.74	7.67±1.22	7.81±0.41
Plasma creatinine (mg/dL)	0.38±0.03	0.32±0.02	0.29±0.02	0.33±0.02
Plasma lithium (mEq/L)	Not detectable	0.13±0.02	0.17±0.05	Not detectable
Plasma Na (mEq/L)	141.6±2.7	143.8±1.0	146.0±0.9	146.0±1.1
Plasma K (mEq/L)	4.4±0.15	4.2±0.25	3.9±0.2	4.5±0.1

Carba, carbamazepine; U<sub>Osm</sub>, urinary Osm; F<sub>e</sub>Na, fraction excretion of sodium in 24 h; F<sub>e</sub>K, fraction excretion of potassium in 24 h.

\*P<0.001 vs. control.

\*\*P<0.01 vs. control.

\*\*\*P<0.05 vs. control.

\*\*\*\*P<0.001 vs. Li.

\*\*\*\*\*P<0.05 vs. Li.

\*\*\*\*\*P<0.001 vs. Li+Carba.

chloride plus carbamazepine group. The densitometric analysis results were: 100.00±6.67 (control), 55.87±5.35 (Li), 75.70±9.56 (Li+Carba) and 99.70±4.73 (Carba).

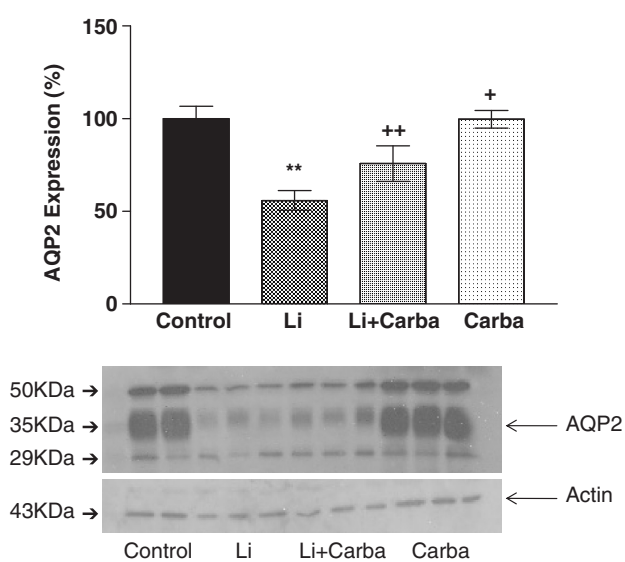
## Discussion

Hyponatraemia reported with the use of carbamazepine has been described as a consequence of a SIADH, although this inappropriate secretion has not been well characterized to date. It has been also suggested that this effect on water metabolism could occur by a carbamazepine-induced increase in the renal sensitivity to the ADH effect [5]. Additionally, this anticonvulsant drug is used to treat patients with diabetes insipidus, but notwithstanding the extensive knowledge regarding its activity in the neurons,

the site of its action as well as the exact nature of its effect on the kidney still have not been fully determined.

Lithium chloride is one of the most important drugs used for management of affective disorders. The principal side effects observed are polyuria and polydipsia as a consequence of a urine-concentrating defect due to vasopressin resistance [12]. It is well known that this nephrogenic diabetes insipidus contributes to the decrease of adherence to the treatment, thus bringing additional morbidity to the patient.

Microperfusion *in vitro*. The osmotic water permeability measured in IMCDs from normal rats demonstrated that, in the absence of ADH, carbamazepine was able to increase water absorption. In all groups of experiments, the dose used was 10<sup>-5</sup> M because it was the lowest dose that caused a reproducible effect since the literature does not show references concerning its use in nephrological studies. Carbamazepine added to the bath solution increased water transport four times circa, showing that this drug has an intrinsic capacity to increase water absorption since these experiments were done in the absence of ADH. In order to identify at what point in the ADH cascade this effect could be occurring, specific inhibitors were used. Firstly, the H8, a PKA cAMP-dependent inhibitor was used showing an inhibition of the carbamazepine effect and demonstrating that its effect is cAMP-dependent. In the second group of experiments, a vasopressin V2 receptor antagonist was used, which also produced a Pf inhibition, evidencing that carbamazepine is acting in the V2 receptor-protein G complex. These results could explain, at least in part, the cases of dilutional hyponatraemia observed in patients using this anticonvulsant drug. Despite the ADH plasma level not having been measured in the present study, the frequent tendency to attribute SIADH as being responsible for the hyponatraemia without measuring the ADH plasma level certainly is not correct anymore. This is an inaccuracy and needs to be re-evaluated. Thirty years ago, in 1978, Stephens *et al.* had already demonstrated that, in patients using carbamazepine, the ADH plasma level was not increased [13].



**Fig. 6.** Western blotting analysis in all groups. Bands 29 and 35–50kDa from AQP2; \*\*P<0.01 (vs. control); +P<0.05 (vs. Li); ++P<0.01 (vs. Li).

*In vivo* studies. To study the carbamazepine effects in animals without the action of ADH in IMCD cells, lithium chloride (to produce nephrogenic diabetes insipidus) was administered, making it possible to demonstrate *in vivo* what was observed *in vitro*.

**Weight.** The results showed a weight reduction after lithium chloride administration, which is in accordance with the literature [7]. In the group that received lithium chloride plus carbamazepine, weights remained low during the time of the experiment. Nothing has been reported in the literature about the effect of carbamazepine on the weight of patients or animals.

**Water ingestion, urinary volume and urinary osmolality.** The data shown in Table 1 reveal that the animals that received lithium chloride presented an increase in urinary volume and in water ingestion, and a decrease in urinary osmolality, showing experimentally in rats the same alterations that were observed in the patients using this drug [6,7]. These alterations were partially recovered by the addition of carbamazepine to the diet. This fact enables us to demonstrate the mechanism of action of the 'carbamazepine therapeutic' effect on NDI [1,2]. The carbamazepine group, however, demonstrated an increase in water ingestion in comparison with the control group. This was an unexpected fact and incomprehensible to us. However, the possibility of carbamazepine, instead of presenting an ability to increase vasopressin secretion, to present a diuretic capacity was conceivable. This hypothesis has, to date, still not been reported and if true, could, at least in part, contribute to producing hyponatraemia, but this finding requires further investigation.

**Aquaporin 2 expression.** IMCDs from normal rats incubated with carbamazepine in the absence of ADH showed an increase in the AQP2 expression, confirming the microperfusion data. Lithium chloride treatment induced a decrease in AQP2 expression, as already demonstrated, since it blocks the ADH action [6]. The association of lithium chloride plus carbamazepine showed a recovery of ~20% of this expression. This result demonstrated that the association of these drugs could bring benefits to the patients in regard to the decrease of polyuria and polydipsia.

It is also well known that hyponatraemia can occur frequently with the association of carbamazepine with other drugs, such as hydrochlorothiazides [14] and fluoxetine [15]. Both of these drugs are known to have the capacity to increase water permeability [16,17]. Carbamazepine association with some other drugs has also been cited as a cause of hyponatraemia [18–20]. Oxcarbazepine, which differs from carbamazepine only by the addition of one oxygen molecule, has the same capacity to produce hyponatraemia [21,22] and probably has the same mechanism of action.

In conclusion, our data showed that carbamazepine has the capacity to increase water permeability, and consequently, the increase of water absorption in the IMCD perfused *in vitro*, acting directly on the ADH V2 receptor–protein G complex and increasing the AQP2 expression. This effect could be responsible, at least in part, for the hyponatraemia observed with the use of this drug. Therefore, the idea that

this hyponatraemia is caused only by a SIADH is not correct anymore.

Finally, our data also showed that this anticonvulsant is an effective drug for decreasing the polyuria and the polydipsia that occur with the use of lithium chloride in the treatment of affective disorders.

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## Renoprotective potency of amifostine in rat renal ischaemia–reperfusion

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### Abstract

**Purpose.** Kidneys from haemodynamically unstable donors may suffer from renal ischaemia–reperfusion (RIR) injury. RIR is associated with reactive oxygen species production that induces inflammation and activates the arachidonic acid (AA) pathway which converts AA into prostaglandin E<sub>2</sub>. Amifostine was investigated for its renoprotective potential in RIR.

**Materials and methods.** The effect of amifostine (25 mg/kg = 910 mg/m<sup>2</sup>) on the COX pathway, enzymatic antioxidant activity, the lipid peroxidation marker MDA, serum creatinine and apoptosis was determined in rats. Kidneys were subjected to 45 min of ischaemia and 1 or 24 h of reperfusion. Control groups (sham: coeliotomy, no ischaemia; r1: 45 min ischaemia/1 h reperfusion; r2: 45 min ischaemia/24 h reperfusion) were administered physiological saline intraperitoneally, and treated groups (E1: 45 min ischaemia/1 h reperfusion; E2: 45 min ischaemia/24 h reperfusion) received amifostine 30 min before reperfusion.

**Results.** Serum creatinine increased in non-treated control rats: r1 vs sham (1.6-fold; P < 0.007), r2 vs sham (2-fold; P < 0.007). Amifostine decreased serum creatinine levels in treated rats: E1 vs r1 (8%; P < 0.0025), E2 vs r2 (44%; P < 0.0025). Amifostine reduced acute tubular necrosis (25%) 24 h after reperfusion: E1 vs r1 (P < 0.004), E2 vs

r2 (P < 0.03) and reduced COX-2 and microsomal prostaglandin E synthase expression: E1 vs r1 (P < 0.03), E2 vs r2 (P < 0.02). Amifostine decreased MDA (P < 0.04) and reduced caspase-3 expression but did not alter enzymatic antioxidant activity after RIR.

**Conclusions.** Amifostine decreased the degree and severity of tubular damage after reperfusion, probably by scavenging oxygen free radicals and attenuating the cytotoxic effects of inflammatory infiltrates and apoptosis.

**Keywords:** amifostine; cyclooxygenase; ischaemia–reperfusion; kidney; reactive oxygen species

### Introduction

Renal ischaemia–reperfusion (RIR) is a complex syndrome involving several mechanisms including renal vasoconstriction, extensive tubular damage and glomerular injury [1,2]. RIR is responsible for renal dysfunction in kidney transplantation and may occur following surgical revascularization of the renal artery, partial nephrectomy, treatment of suprarenal aortic aneurysms and after cardiac surgery [3,4]. The mechanisms involved in RIR injury include an-