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L-Carnitine is an osmotic agent suitable for peritoneal dialysis

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Excessive intraperitoneal absorption of glucose during peritoneal dialysis has both local cytotoxic and systemic metabolic effects. Here we evaluate peritoneal dialysis solutions containing L-carnitine, an osmotically active compound that induces fluid flow across the peritoneum. In rats, L-carnitine in the peritoneal cavity had a dose-dependent osmotic effect similar to glucose. Analogous ultrafiltration and small solute transport characteristics were found for dialysates containing 3.86% glucose, equimolar L-carnitine, or combinations of both osmotic agents in mice. About half of the ultrafiltration generated by L-carnitine reflected facilitated water transport by aquaporin-1 (AQP1) water channels of endothelial cells. Nocturnal exchanges with 1.5% glucose and 0.25% L-carnitine in four patients receiving continuous ambulatory peritoneal dialysis were well tolerated and associated with higher net ultrafiltration than that achieved with 2.5% glucose solutions, despite the lower osmolarity of the carnitine-containing solution. Addition of L-carnitine to endothelial cells in culture increased the expression of AQP1, significantly improved viability, and prevented glucose-induced apoptosis. In a standard toxicity test, the addition of L-carnitine to peritoneal dialysis solution improved the viability of L929 fibroblasts. Thus, our studies support the use of L-carnitine as an alternative osmotic agent in peritoneal dialysis.

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Removal of excess fluid is a key component of dialysis treatment that is achieved in continuous ambulatory peritoneal dialysis (CAPD) by addition of an osmotic agent to the solution. The generalized use of glucose (molecular weight 180 Da) as an osmotic driving force is based on its efficacy, low cost, and an acceptable safety profile. However, exposure to massive amounts of glucose during the course of CAPD is increasingly considered as detrimental for the peritoneal membrane (PM).[1] Glucose absorption during the exchange causes a progressive dissipation of the osmotic gradient,[2] and may play a distinct role in longitudinal changes to the PM structure and function.[3] Furthermore, excessive intraperitoneal absorption of glucose and its intermediates has both potential local cytotoxic effects and systemic metabolic effects because of carbohydrate load, caloric uptake, and hyperglycemia.[4]

Thus, several substances have been examined as alternative osmotic agents to glucose for peritoneal dialysis (PD). However, only two such agents have had any reasonable clinical success: icodextrin and aminoacids. The colloidal osmotic agent icodextrin achieves sustained ultrafiltration (UF) in an iso-osmolar solution and is indicated for use during a single long dwell. Aminoacid-based solutions have the advantage of replacing glucose in the solution and improving the protein nutrition and metabolic status of PD patients. The benefits of glucose sparing offered by above formulations, either alone or in combination, have recently been reported.[5–7] However, it should be noted that both icodextrin and aminoacids can only replace 30–50% of daily glucose absorption.[4] Moreover, clinical application of aminoacids is limited to a single daily exchange because of the potential risk of acidosis and azotemia-related side effects,[8] whereas icodextrin has also proven to have limitations for prolonged use.[9] In addition, use of such solutions brings additional costs.

L-Carnitine is a naturally occurring compound best known for its involvement in the mitochondrial oxidation of long-chain fatty acids.[10] L-Carnitine is a key substrate in a family of enzymes known as carnitine acyltransferases, which reversibly

transfer activated acyl units between carnitine and coenzyme A to preserve coenzyme A homeostasis for the broad range of acyl-trafficking activities that are crucial for intermediary metabolism and cell regulation.[11,12]

L-Carnitine (molecular weight 161.2 Da) is highly water soluble, chemically stable in aqueous solutions (pH 2–8) up to temperatures of 200 °C,[13] and it has been shown to posses osmotic properties in different biological systems.[14,15] In addition, L-carnitine has been extensively used in therapy and has shown an excellent safety profile, even when systemically administered in high dosages.[16,17] Moreover, recent findings show that a PD solution containing L-carnitine seems to be more biocompatible than standard glucose solutions or those containing icodextrin in terms of mesothelial and vascular changes.[18] Given its physicochemical and drug properties, we have conducted *in vivo* studies, in rat and mouse models and CAPD patients, as well as *in vitro* experiments, to evaluate the functional effects of PD osmotic solutions containing L-carnitine.

RESULTS

Animal studies

Rat studies: L-carnitine and D-glucose generate the same UF. Experiments were conducted to evaluate the transport ability of various PD solutions containing either D-glucose or L-carnitine at 1.5, 2.5, and 4.25% (weight/volume). The percent changes in fluid recovered from rats submitted to PD are shown in Figure 1. As expected, a dose-dependent increase in fluid recovery was observed with increasing concentrations of the osmolyte, D-glucose or L-carnitine, present in the PD solution. The figure also shows that at the various concentrations of the osmolyte used, fluid recovery plateaued after 2 h of the PD solution residing in the peritoneum, producing no further increase in fluid recovery at later dwell times (4 and 6 h). At each concentration used, the ability of L-carnitine to generate UF was similar to that of glucose.

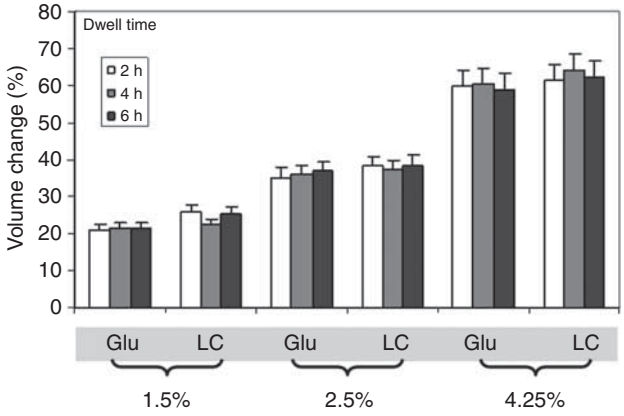

Figure 1 | Acute peritoneal dialysis (PD) studies in normal rats. The figure shows the percentage change in peritoneal fluid recovered from rats ($n = 6$ for each treatment) submitted to PD solution containing glucose (Glu) or L-carnitine (LC) at indicated concentrations.

Mouse studies: water and small solute transport. We first investigated the effects on transport parameters of two types of dialysate, standard glucose-based dialysate (Dianeal 3.86%, Baxter Healthcare, Nivelles, Belgium) and L-carnitine-based dialysate (L-carnitine amount, 214 mmol/l, equimolar to glucose present in Dianeal 3.86%), in standard C57 mice using a 2-h PD exchange (Table 1). This protocol induced a progressive increase in the dialysate-to-plasma ratio for urea, a progressive re-absorption of glucose from the dialysate, a fall in the dialysate-to-plasma ratio of sodium during the first 30 min of the dwell (sodium sieving), and a net UF as previously reported.[19] Exposure to L-carnitine 214 mmol/l generated same UF and sodium sieving than the standard 3.86% glucose dialysate (Table 1), with similar parameters of small solute transport (data not shown). In further studies, exposure to two different concentrations of L-carnitine in glucose (final molarity identical to 3.86% glucose) generated the same UF than the standard 3.86% glucose dialysate (Table 2), again with similar parameters of small solute transport (Supplementary Figure S1 online).

Studies in the aquaporin-1 (AQP1)-deficient mice (Table 3) demonstrated that ~50% of the UF generated by L-carnitine dialysate reflected facilitated water transport through the ultrasmall pores. This AQP1-mediated UF was similar with 3.86% glucose and L-carnitine 214 mmol/l. The urea transport was also similar with the two types of dialysates and was not influenced by the deletion of AQP1 (Supplementary Figure S2 online).

Clinical studies in PD patients

Following experiments in the animal model, an exploratory study was carried out in four adult CAPD patients. The

Table 1 | Transport parameters in C57 mice exposed to equimolar amount of glucose or L-carnitine

	Sodium sieving (%)	Net ultrafiltration (µl/g BW)
Glucose 3.86% (214 mmol/l)	4.5 ± 0.8	30.6 ± 1.1
L-Carnitine (214 mmol/l)	2.7 ± 0.8	28.5 ± 1.7

Abbreviation: BW, body weight.
Sodium sieving: fall (%) in the dialysate-to-plasma ratio of sodium during the first 30 min of the dwell.
Results are mean ± s.e.m. values; n=5 mice per group.

Table 2 | Transport parameters in C57 mice exposed to glucose and two different L-carnitine concentrations plus glucose

	Net ultrafiltration (µl/g BW)	D/P urea T120
Glucose 3.86% (214 mmol/l)	46 ± 1.9	0.74 ± 0.01
Glucose (199 mmol/l)+L-carnitine (15 mmol/l)	48 ± 1.6	0.74 ± 0.02
Glucose (171 mmol/l)+L-carnitine (43 mmol/l)	47 ± 2.5	0.73 ± 0.01

Abbreviations: BW, body weight; D/P, dialysate/plasma ratio.
The final molarity of the glucose-carnitine mixture solutions was 214 mmol/l.
Results are mean ± s.e.m. values; n=6 mice per group.

Table 3 | Effect of AQP1 deletion on ultrafiltration

	Ultrafiltration (μl/g BW)		
	Aqp1 WT	*Aqp1* KO	Δ (%)
Glucose 3.86% (214 mmol/l)	33.5 ± 2.1	12.0 ± 1.6*	64%
L-Carnitine (214 mmol/l)	28.8 ± 1.0	13.5 ± 0.9*	53%

Abbreviations: AQP1, aquaporin-1; BW, body weight; KO, knockout mice; WT, wild-type mice.
Results are mean ± s.e.m. values; n=5 mice per group.
*P < 0.05 vs WT mice.

experimental protocol consisted of the use for 5 consecutive days of a mixture solution (1.5%, 75.5 mmol/l, glucose plus 0.25%, 15.5 mmol/l, L-carnitine) replacing a 2.5% (126.1 mmol/l) glucose-based solution for the nocturnal dwell.

In terms of safety and tolerability, no patient referred the appearance or worsening of any of the complaints listed in Supplementary Table S1 online. There were no clinically significant changes from baseline in vital signs or electrocardiogram findings (Supplementary Table S2 online) or in any laboratory variable (Supplementary Table S3 online). Physical examination, concomitant illnesses, and pharmacological treatments were unmodified.

Parameters of dialysis efficiency including creatinine clearance and urea Kt/V proved to be stable (Supplementary Table S4 online) throughout the 5 consecutive days of L-carnitine treatment. Fluid status, body weight, diuresis, and total UF also proved to be stable (Supplementary Table S4 online). The net drained UF volume from nocturnal dwell exchange with L-carnitine-added solution, however, was higher than that obtained by 2.5% glucose solution (Figure 2a). When the mean peritoneal nocturnal UF during the 5 days of L-carnitine treatment was compared with the 5 days before (use of 2.5% glucose), an increase was found (Figure 2b).

Peritoneal equilibration test (PET) analysis at the end of L-carnitine administration did not differ from baseline evaluation (Supplementary Table S4 online).

Kinetics of L-carnitine absorption and excretion. As L-carnitine is a relatively small and highly soluble molecule, we evaluated the kinetics whereby it appeared in the bloodstream and was excreted through the dialysate (ultrafiltrate) and urine over 5-day nocturnal use of L-carnitine-enriched PD solution. As expected, after the first administration of L-carnitine, plasma L-carnitine levels at the end of the long dwell rose from 0.05 to ~0.7 mmol/l (Figure 3a). The next administrations of L-carnitine led to a further rise in plasma levels, which was less steep than the previous ones.

Because L-carnitine administration in mammals is invariably associated with an increase of its major metabolic congeners, we also determined plasma acetyl-carnitine (ALC) levels. Figure 3a shows the plasma ALC plasma profile obtained at the end of each long dwell throughout the five L-carnitine administrations. The plasma ALC profile was mainly characterized by a smooth increase in its plasma levels.

The profile of total L-carnitine recovered in drained dialysate and urine (Figure 3b) indicates that after each subsequent

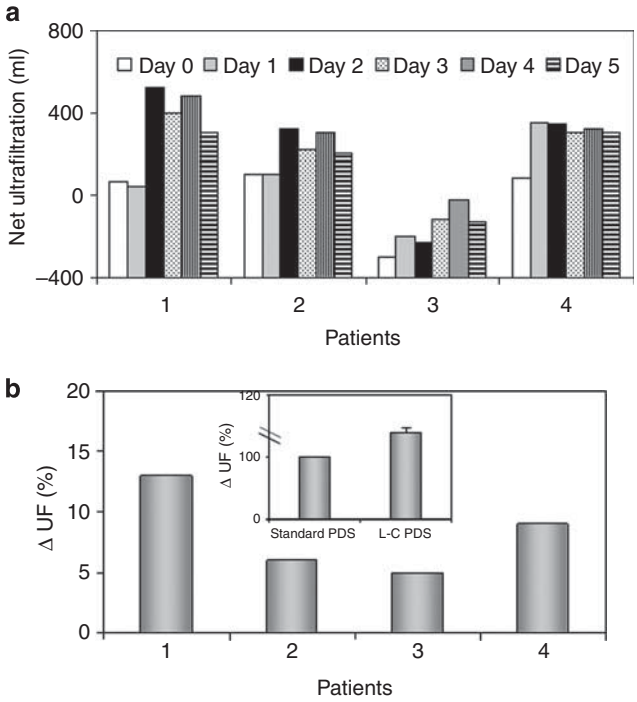

Figure 2 | Peritoneal ultrafiltration in PD patients. (**a**) Net peritoneal nocturnal ultrafiltration after long dwell with 2.5% glucose (day 0) and 1.5% glucose plus 0.25% L-carnitine (day 1 to day 5) in four continuous ambulatory peritoneal dialysis (CAPD) patients. (**b**) Difference in mean peritoneal nocturnal ultrafiltration (UF) during 5 consecutive days of mixture solution (glucose 1.5% + L-carnitine 0.25%) compared with the previous 5 days of standard solution (2.5% glucose). The inbox summarizes the results (expressed as mean ± s.e.m.) of the whole group of patients, when compared with the mean value pretest (set at 100%).

L-carnitine administration, more total carnitine is recovered (drained dialysate plus urine), and more total carnitine is recovered in drained dialysate than urine. In drained dialysate, after the first carnitine administration, 65% of carnitine was recovered after the long dwell and 35% in the three short dwells. In the following L-carnitine administrations, the percentage of carnitine recovered in the long dwell linearly decreased and, at the end of the treatment (fifth L-carnitine administration), was close to 50%. According to the amount of L-carnitine recovered in the drained dialysate of the long dwell, the percentages of L-carnitine absorbed were on average 85.5, 80, 75, 72, and 77% after the first, second, third, fourth, and fifth carnitine administration, respectively. The percentage of L-carnitine recovered in the short dwells increased linearly from 35% at the beginning to up to 50% at the end of the treatment. On average, >80% of total L-carnitine recovered in drained dialysate and urine was represented by free carnitine.

In vitro studies

Effects of L-carnitine on cell viability and AQP1 expression in human endothelial cells. As chronic high glucose exposure induces long-term detrimental microvascular tissue effects,[20,21] we decided to evaluate the effects of several doses of L-carnitine

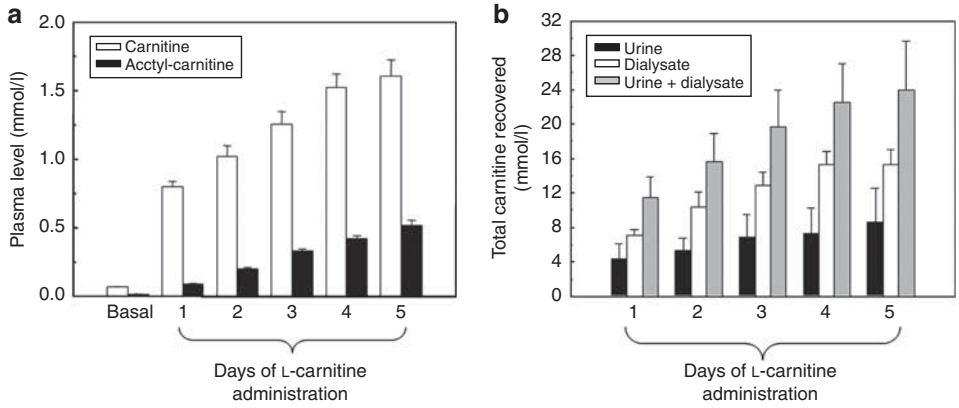

Figure 3 | L-Carnitine and acetyl-carnitine kinetics in PD patients. (**a**) Plasma levels of L-carnitine and acetyl-carnitine at baseline (basal) and during 5 days of mixture solution (glucose 1.5% + L-carnitine 0.25%) used for nocturnal exchange in four continuous ambulatory peritoneal dialysis (CAPD) patients. (**b**) Recovery of total L-carnitine (free L-carnitine plus acetyl-carnitine) in urine and drained dialysate during 5 days of mixture solution (glucose 1.5% + L-carnitine 0.25%) used for nocturnal exchange in four CAPD patients.

on cell viability (in the presence or absence of high glucose concentrations) and on glucose-induced early apoptosis in cultured human umbilical vein endothelial cells (HUVECs). Figure 4a shows that D-glucose (30 mmol/l) led to an increase in early apoptotic cells, as measured by the annexin V-propidium iodide assay (9.2 ± 0.6 vs 4.6 ± 2.7%, D-glucose vs control, $P<0.05$). On the contrary, when compared with control cells, L-carnitine alone (2–30 mmol/l) did not affect the percentage of early apoptotic cells. Note that the addition of L-carnitine (2–30 mmol/l) to HUVECs prevented glucose-induced early apoptosis (6.7 ± 0.6, 6.9 ± 0.9, and 5.9 ± 0.6 vs 9.2 ± 0.6%, respectively, for 2, 10, and 30 mmol/l of L-carnitine vs D-glucose, $P<0.05$) and significantly increased cell viability (Figure 4b, $P<0.05$). Osmotic control mannitol (30 mmol/l) was without any effect on apoptosis and cell viability.

At L-carnitine concentrations >30 mmol/l, some toxicity on HUVEC viability and induction of apoptosis was observed, although to a lower extent than glucose (Supplementary Figure S3 online).

As peritoneal capillaries contain the ultrasmall pores AQP1[22,23] and because we observed that *in vivo* L-carnitine increased UF, we evaluated whether the expression of AQP1 in HUVECs could be modulated by addition of low doses of L-carnitine (2–30 mmol/l) and/or D-glucose (30 mmol/l). As shown by western blot analysis (Figure 5), the exposure of HUVECs to a high glucose medium resulted in a significant decrease in AQP1 protein levels ($P<0.001$). The addition of L-carnitine (2–30 mmol/l) to D-glucose significantly reverted the inhibitory effect of glucose on AQP1 protein expression ($P<0.05$). Of note, L-carnitine alone (2 and 10 mmol/l) significantly increased AQP1 protein levels ($P<0.001$). Osmotic control (mannitol 30 mmol/l) had no effect on the AQP1 protein level.

Effects of L-carnitine on the viability of murine fibroblasts. Cultured murine fibroblast L929 have previously been employed to demonstrate the general toxicity of commercial PD solutions;[24] thus, we evaluated the L929 growth (3H-TdR incorporation) and viability (MTT test) in the presence of

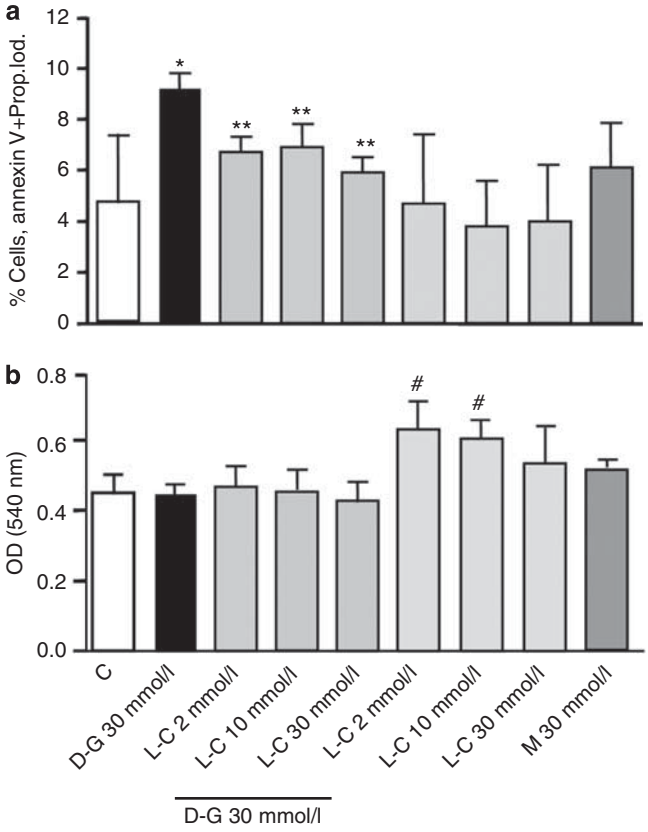

Figure 4 | Effects of L-carnitine on human endothelial cell viability. (**a**) Flow cytometry analysis of endothelial cell apoptosis measured by the annexin V-propidium iodide (Prop.Iod.) assay and (**b**) the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. C, Control; D-G, D-glucose (30 mmol/l); L-C, L-carnitine (2–30 mmol/l); M, mannitol (30 mmol/l). *$P<0.05$ D-glucose (30 mmol/l) vs Control, **$P<0.05$ L-carnitine (2–30 mmol/l) plus D-glucose (30 mmol/l) vs D-glucose (30 mmol/l), #$P<0.05$ L-carnitine (2 and 10 mmol/l) vs Control. Results are mean ± s.d. values from three independent experiments with different batches of human umbilical vein endothelial cells (HUVECs). OD, optical density.

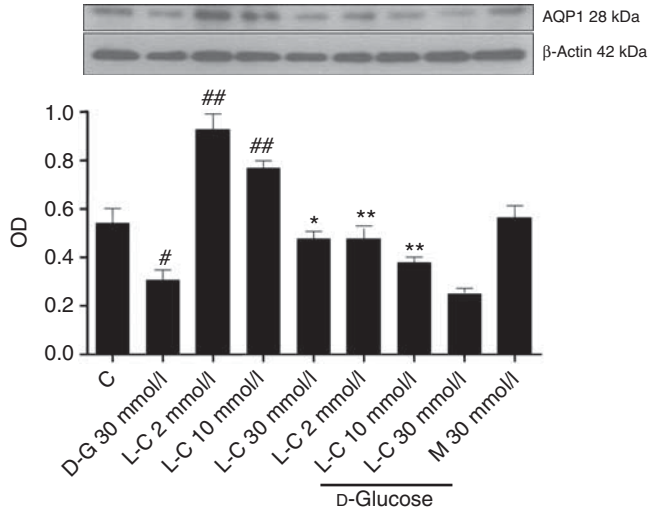

Figure 5 | Aquaporin-1 (AQP1) protein expression in human umbilical vein endothelial cells (HUVECs) stimulated by D-glucose (30 mmol/l) and/or L-carnitine (2–30 mmol/l), and mannitol (30 mmol/l). C, Control; D-G, D-glucose (30 mmol/l); L-C, L-carnitine (2–30 mmol/l); M, mannitol (30 mmol/l). $^{\#}P<0.001$ D-glucose (30 mmol/l) vs Control; $^{\#\#}P<0.001$ L-carnitine (2 and 10 mmol/l) vs Control; $^{*}P<0.05$ L-carnitine (30 mmol/l) vs D-glucose (30 mmol/l); $^{**}P<0.05$ L-carnitine (2 and 10 mmol/l) vs D-glucose (30 mmol/l). Results are mean ± s.d. values from three independent experiments with different batches of HUVECs.

standard 1.5% (Figure 6a) and 2.5% (Figure 6b) glucose-based PD solutions, with or without the presence of L-carnitine at different concentrations (0.25–30 mmol/l). The addition of each dose of L-carnitine to either 1.5 or 2.5% glucose significantly improved L929 viability, whereas cell growth was not significantly modified by addition of L-carnitine (Figure 6a and b). It should be noted that as our PD solutions were filter sterilized, they are virtually clean of any glucose degradation products.

DISCUSSION

In PD therapy, several observations have highlighted the importance of a capacity for UF across the PM on patient outcome.[25,26] Failure of UF capacity is the most frequent abnormality in patients on long-term PD, and is the main reason for technical failure.[27] Awareness of the potentially unfavorable effects of hypertonic glucose to the PM[28] has stimulated the development of glucose-sparing strategies able to provide an efficacious UF profile.[4]

The results of this study suggest that L-carnitine has potential use in the PD solution as a new osmotic agent (with a potential glucose-sparing effect). In animal studies, L-carnitine instilled into the peritoneal cavity showed a dose-dependent osmotic capacity comparable with that of glucose (Figure 1 and Tables 1 and 2). In addition, studies in the *Aqp*1 mice demonstrated that the UF generated by equimolar L-carnitine dialysate was comparable with standard 3.86% glucose (Table 3). Moreover, L929 experiments showed that, besides its *in vivo* osmotic properties, L-carnitine is able to significantly increase cell viability, thus indicating its potential protective role on PM (Figure 6). This is in line with several scientific accounts to the effect that, at comparable concentrations, L-carnitine is able to counteract the proapoptotic processes in various different experimental models.[29–32]

Importantly, L-carnitine displayed osmotic properties in clinical studies carried out in stable CAPD patients. Our data suggest that after nocturnal exchange with a dialysis bag containing glucose 1.5% (75.5 mmol/l) plus L-carnitine 0.25% (15.5 mmol/l), the drained UF volume was higher than that obtained with a standard 2.5% (126.1 mmol/l) glucose-based dialysis solution despite the lower osmolarity of the experimental solution (362 vs 398 mOsm/l, calculated), thereby indicating the capacity of L-carnitine for UF across the peritoneum.

The capacity shown by L-carnitine for removing fluid from the peritoneal cavity might be related to its chemical–physical properties. L-Carnitine has a slightly lower molecular weight than glucose and, more important, it is a highly charged compound, a feature that may affect osmotic processes across the PM. Indeed, as the plasma L-carnitine levels rise, the dialysate to plasma L-carnitine concentration gradient and thus diffusion gradient will fall. The resultant reduction in the disappearance rate of L-carnitine will mean that the osmotic properties of the fluid will be better maintained, resulting in better UF (without any change in membrane function on the PET). This reasoning seems to be corroborated by the fact that toward the end of the study period the amount of L-carnitine recovered in the drained dialysate of the long and short dwells was almost twice as much than that recovered at the beginning of the study (Figure 3).

Alternatively, but not mutually exclusive, the UF capacity of L-carnitine might also occur through a favorable action on the water channel AQP1, which is distributed throughout the endothelium lining capillaries, venules, and small veins of the peritoneum.[22,23] According to the three-pore model, the major transport barrier of the PM is the capillary endothelium, which contains ultrasmall pores (<3 Å) that facilitate the osmotic transport of water. Computer simulations have predicted that these ultrasmall pores account for ~50% of the UF.[33] Recently, the use of AQP1 knockout mice provided a direct demonstration for the role of water channels as the ultrasmall pores mediating approximately half of the UF during a dwell with crystalloid glucose.[34] Our studies took advantage of this mouse model[35] to demonstrate that L-carnitine-based dialysates generate a net UF that is similar to that generated by 3.86% glucose and that, like in classical crystalloid osmosis generated by hypertonic glucose, approximately half of this UF is mediated by AQP1 (Table 3).

We have also observed that in cultured HUVECs, high glucose causes a significant decrease in the AQP1 protein levels and a parallel increase in the early apoptotic cell percentage. Interestingly, the addition of L-carnitine (2 and

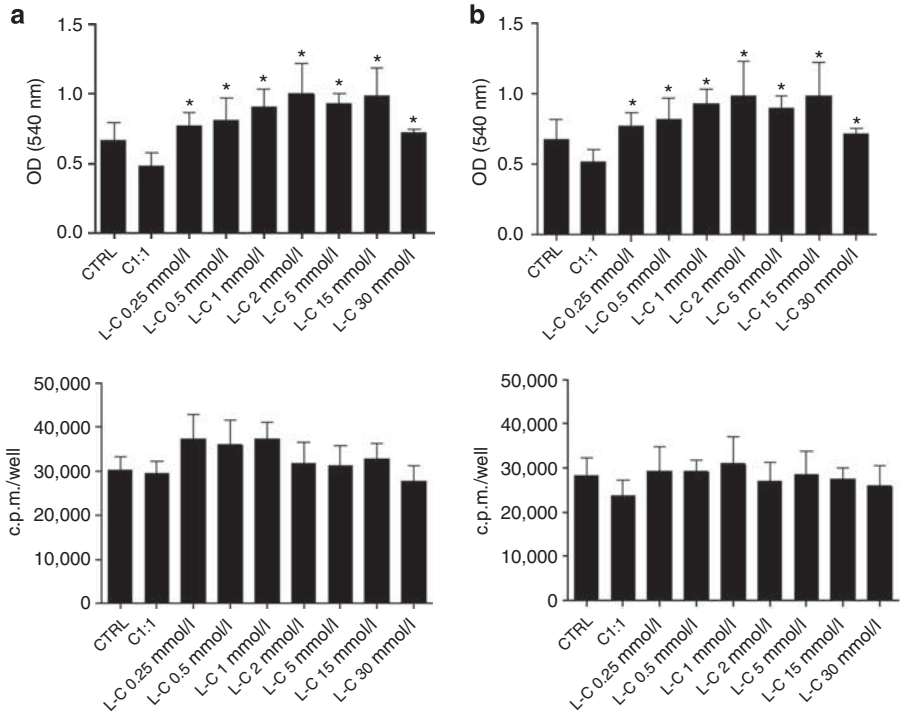

Figure 6 | Effect of L-carnitine (0.25–0.5–1–2–5–15–30 mmol/l) on L929 growth and viability. L-Carnitine was diluted in peritoneal dialysis (PD) solution containing (**a**) 1.5% glucose or (**b**) 2.5% glucose. C, Control, modified Eagle's medium (MEM); c.p.m., counts per minute; C1:1, PD solution/MEM (v/v); L-C, L-carnitine. *$P < 0.01$ L-carnitine (0.25–30 mmol/l) vs C1:1. Results are mean ± s.d. values from three independent experiments with different batches of human umbilical vein endothelial cells (HUVECs).

10 mmol/l) to D-glucose significantly reversed the inhibitory effect of glucose on both AQP1 levels and apoptosis.

Although the D-glucose effect on early apoptosis in cultured HUVECs was in accordance with previous data,[36] its effect on AQP1 protein level is apparently at variance with a previous report[37] demonstrating endothelial-increased glucose-stimulated AQP1 expression. This apparent discrepancy may be because of the different experimental conditions: acute stimulation with very high glucose concentrations (from 50 up to 200 mmol/l) performed in EA.hy 926 cell line,[37] and chronic stimulation with 30 mmol/l glucose performed in HUVECs here.

Use of LC in the PD fluid was safe and well tolerated in all patients, in keeping with the excellent safety profile in hemodialysis (HD) patients. As L-carnitine in mammals does not get further metabolized, although it may convert to acyl-chain esters via acyl-carnitine family transferases,[11,12] we decided to measure how L-carnitine and ALC appear in the blood and how they are excreted in the UF and urine. At the end of the five consecutive L-carnitine administrations, free L-carnitine and ALC levels in blood were 1.6 and 0.5 mmol/l, respectively. It was also observed that at the end of each L-carnitine administration, the increase in L-carnitine and ALC blood levels was less steep than the previous one. The lag phase in the appearance of ALC in plasma seems to be driven by the fact that L-carnitine has to become available most likely in the muscle compartment, where it may take part in the mitochondrial reaction catalyzed by carnitine acetyl-transferase.[12] Although these concentrations are significantly higher than those present in healthy subjects, very high plasma carnitine exposure in HD patients treated with L-carnitine for a prolonged period of time has been safely achieved.[16] As expected, L-carnitine and ALC are efficiently disposed of through UF and urine, and their excretion kinetics clearly indicate that after each consecutive L-carnitine administration, more L-carnitine and its acetyl-ester are excreted. The total amount of L-carnitine recovered in both UF and urine at the end of the study (24 h after the last administration) is 93.2 ± 13.3 mmol, whereas the total amount of L-carnitine administered is 155 mmol. This implies that ~40% of the L-carnitine administered has been retained in the body. Our data also indicate that at the end of the last L-carnitine administration, the percentage of L-carnitine retained is ~20% (Figure 3b), and even less L-carnitine would have been retained if we had prolonged the L-carnitine administration. In other words, because of the fall of the dialysate to plasma carnitine concentration gradient, less and less carnitine will be absorbed from the peritoneal cavity during successive long dwells, which would further limit the increase of plasma carnitine levels. Moreover, we have also noticed an inverse relationship between the two main carnitine excretory routes (peritoneum and urine): those patients excreting more carnitine via the peritoneum excreted less carnitine in the urine and vice versa (unpublished observations, A. Arduini and M. Bonomini). Thus, even in anuric patients, carnitine would be excreted via the

peritoneum. Of note, L-carnitine and its acyl-esters can be excreted through the biliary route,[38,39] although the extent of this excretory pathway in PD patients is not known.

As excessive glucose exposure in PD patients has many potential disadvantages,[4] strategies designed to reduce/abrogate glucose-associated toxicity form one of the modern goals of PD therapy. L-Carnitine use in the PD solution might represent a new option. The effect may be achieved by a reduction in the glucose concentration in the dialysate not altering the UF profile through the addition of L-carnitine. Furthermore, many reports have shown that L-carnitine has a favorable effect on glucose metabolism (rev. in Arduini *et al.*[40]). The action of L-carnitine on glucose metabolism indicates that significant exposure of insulin target organs to physiological concentrations of L-carnitine may improve the altered glucose homeostasis in PD patients, thereby improving insulin sensitivity. In a small randomized, double-blind, placebo-controlled study, intravenous L-carnitine administration in HD patients favorably affected insulin resistance and protein catabolism in non-diabetic HD patients.[41]

Our study has some obvious limitations. *In vivo* results were obtained in short-term clinical studies involving a small number of patients. Thus, larger randomized trials of longer duration are required before any definitive conclusion can be drawn as to the possible use of L-carnitine in PD. Appropriate concentrations of the dialysate mix components also remain to be defined. Finally, the effects of L-carnitine administration to anuric patients (all patients in our studies had preserved diuresis) are at present unknown. Notwithstanding this, the good tolerability and the potential local and systemic advantages of L-carnitine use in the PD solution are in our opinion worthy of further investigation. Long-term studies with L-carnitine-based solution bags, which are in progress (ClinicalTrials.gov Identifiers: NCT00755456 and NCT00755404), will help to clarify and justify the use of L-carnitine in PD therapy.

MATERIALS AND METHODS

Materials

Phosphate-buffered saline, glutamine, fetal calf serum, M199 endothelial growth medium, Dulbecco's modified Eagle's medium, modified Eagle's medium, 0.05% trypsin/0.02% EDTA, gentamycin, and nonessential aminoacids were purchased from Mascia Brunelli (Milan, Italy); heparin, collagenase, gelatin, endothelial cell growth factor, D-glucose, D-mannitol, L-carnitine, and the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were from Sigma Chemical (St Louis, MO). Anti-AQP1 was from Santa Cruz Biotechnology (Santa Cruz, CA). [^{3}H]-Thymidine was purchased from PE Applied Biosystems (Foster City, CA) Tissue-culture disposables were purchased from Hiwaki Glass (Tokyo, Japan).

Animal models

Animal care and treatment were conducted in conformity with the guide for the care and use of laboratory animals of the National Institutes of Health, and the experiments were approved by the local ethics committee.

The composition of PD solutions for rat studies was as follows: NaCl 5.786 g/l, CaCl2 · 2H2O 0.257 g/l, MgCl2 · 6H2O 0.102 g/l, sodium D/L-lactate 3.925 g/l, and anhydrous D-glucose 15.0 g/l (1.5%, 83.3 mmol/l), 25.0 g/l (2.5%, 138.8 mmol/l), or 42.5 g/l (4.25%, 236.1 mmol/l), or L-carnitine (inner salt) 15.0 g/l (1.5%, 93.1 mmol/l), 25.0 g/l (2.5%, 155.2 mmol/l), or 42.5 g/l (4.25%, 263.9 mmol/l). PD solutions were freshly prepared, buffered at pH 5.5 with HCl, and sterilized by filtration through 0.45 μm pore-size membranes.

Male Sprague-Dawley rats weighing 500–600 g (Charles River, Calco, Italy) were maintained on a standard diet with water *ad libitum*. Rats were placed on an operating table at a controlled temperature and anesthetized with an intraperitoneal injection of inactin (100 mg/kg). In order to cannulate the left jugular vein by a medical silicone tube, anesthetized rats were submitted to tracheostomy. The animals were then infused with a saline solution at a rate of 2.3 ml/h throughout the period of the experiment. Next, 15 ml of preheated (37 °C) dialysis solution was instilled into the peritoneal cavity 1 h after administration of the anesthetic. The amount of fluid injected was determined by weighing the syringe before and after injection of the fluid, using an electronic scale. At the end of each time period (2, 4, and 6 h), incisions were made in the abdomens of the rats with an acusector and all the fluid present in the peritoneum was aspirated with a 1 ml syringe. In addition, the intestines were carefully shifted from the abdominal cavity to aspirate the residual fluid present on the dorsal wall. The fluid recovered was placed in a beaker and weighed. The change in weight compared with time 0 was taken as the amount of fluid recovered from the peritoneal solution instilled.

Experiments were also performed on adult male C57 BL/6J mice (Iffa Credo, Brussels, Belgium) and wild-type and knockout *Aqp1* mice.[42] The mice were aged 8 to 12 weeks. All animals had access to appropriate standard diet and tap water *ad libitum*. The first series of experiments were performed on the C57 mice, in order to compare the net UF generated by Dianeal 3.86% and a PD solution containing L-carnitine in amount equimolar (214 mmol/l) to the glucose present in the Dianeal 3.86%. In further experiments, the net UF generated by Dianeal 3.86% (osmolality 512 mOsm/kg) was compared with that obtained with two mixtures of L-carnitine with glucose of similar osmolality: glucose 199 mmol/l + L-carnitine 15 mmol/l (520 mOsm/kg), and glucose 171 mmol/l + L-carnitine 43 mmol/l (494 mOsm/kg). The composition of L-carnitine-based PD solution was as follows: NaCl 5.786 g/l, CaCl2 · 2H2O 0.257 g/l, MgCl2 · 6H2O 0.102 g/l, sodium D/L-lactate 3.925 g/l, and L-carnitine (inner salt) at the above indicated concentration. The solution was freshly prepared, buffered at pH 5.5 with HCl, and sterilized by filtration through 0.45 μm pore-size membranes.

In a second round of experiments, we used *Aqp1* mice to determine the role of water channels in the UF generated by the various dialysates.

A PET was used to investigate peritoneal transport parameters in mice as previously described.[19] Briefly, after anesthesia with ketamine and xylazine, mice were placed on a thermopad at 37 °C and catheterized for blood pressure monitoring (right common carotid artery) and saline infusion (right jugular vein, 0.9% NaCl, 0.3 ml/h). After 30 min, a silicon catheter (Venflon 22 GA; Baxter) was inserted into the peritoneal cavity and 2.0 ml of the appropriate dialysate was instilled. Blood and dialysate samples (50–100 μl) were taken from carotid artery and the PD catheter at time 0 and at 30, 60, and 120 min of dwell time. Hematocrit was measured before PD exchange. At the end of the dwell, the dialysate was recovered from the peritoneal cavity and net UF was calculated.[43] The mice were

exsanguined and urea, glucose, sodium, and total protein were assayed using a Kodak Ektachem DT60 II and DTE II analyzers (Eastman Kodak Company, Rochester, NY).

Clinical studies

Clinical studies were performed in prevalent non-anuric uremic patients established on a CAPD regimen with standard solutions for at least 3 months, presenting at the PD Center of the University of Chieti. Inclusion criteria at baseline evaluation included stability of PD efficiency parameters over the last 3 months, as well as a dialysate/plasma creatinine ratio between 0.50 and 0.81 and a dialysate/plasma glucose ratio between 0.26 and 0.49 at PET. Main exclusion criteria were any unstable clinical condition or peritonitis episodes in the 3 months before baseline, diabetes mellitus, presence of infectious conditions, a history of congestive heart failure, or malignancy.

The procedures were in accordance with the ethical standards of the Institutional Committee on Human Experimentation and with the Declaration of Helsinki Principles (as revised in 1983). After approval of the protocol by the institutional review board, signed informed consent form was obtained from each participating patient.

The clinical study was designed to evaluate dialysis safety, tolerability, and efficiency of a nocturnal glucose-based solution (1.5% glucose, 75.5 mmol/l, Dianeal; Baxter Healthcare) with 5 g L-carnitine (0.25%, weight/volume; 15.5 mmol/l) added, versus a PD solution containing glucose only (2.5% glucose, 126.1 mmol/l, Dianeal; Baxter). Carnitine (Carnitene, Sigma Tau IFR, Pomezia, Italy) was added *via* the access port of the PD bag under a laminar flow hood. The reason for choosing the amount of 5 g L-carnitine was mainly dictated by safety considerations, with HD and immunodeficient patients having been safely treated with intravenous injections of L-carnitine at dosages close to what we have used here.[16,17] A group of four patients (3 male and 1 female, mean age 61.6 ± 11.9 years) receiving three 2-L exchanges of 1.5% glucose for the daytime dwells, and one 2-L bag of 2.5% glucose for the long night dwell, were enrolled. Patients were asked to keep a daily record of body weight, diuresis, and peritoneal UF during 5 days before treatment. Then, they were switched for 5 consecutive days onto the above-described glucose-based solution with L-carnitine added for the nocturnal exchange, with diurnal exchanges being unmodified.

The following parameters were evaluated:

(a) Safety and tolerability parameters: appearance/worsening of possible complaints (listed in Supplementary Table S1 online), physical examination, body weight, vital signs, concomitant illnesses and pharmacological treatments, electrocardiogram, and laboratory parameters (biochemical tests performed with the standard techniques);
(b) Fluid removal: this consisted of daily urine and peritoneal UF. To calculate peritoneal UF, at each dwell the fresh PD bag was weighed before and after the flush before fill procedure, to correct for the flush before fill rinsing volume (being not used a fixed volume) and for possible over- or under-fill of the bag. From the latter weight, the volume of infused PD solution was obtained by subtracting the weight of the empty bag. The volume of the drained dialysate was measured by weighing the drainage bag and then subtracting the weight of the empty bag. Peritoneal UF was calculated (ml) as drained (ml)–infused (ml) volume. Total daily removal was calculated as the sum of daily urine and UF;
(c) PD efficiency: at baseline and during each day of L-carnitine treatment, creatinine clearance (residual renal clearance + dialysate clearance) and urea Kt/V were determined to assess dialysis adequacy; a PET was performed to assess peritoneal permeability at baseline and at the end of the study;
(d) Free L-carnitine and acyl-carnitine esters in blood (plasma), peritoneal solution drained out, and urine. Free L-carnitine and its acyl-chain esters (total L-carnitine level) were measured by high-performance liquid chromatography/mass spectrometry.[44] More than 99% of total L-carnitine levels in plasma, UF, and urine is represented by the sum of free L-carnitine plus ALC The total L-carnitine level was determined in the long dwell UF plus the three short dwell UFs, as well as in urine collected from the beginning of the long dwell for 24 h.

HUVEC experimental protocol

HUVECs were obtained and grown as previously described[45] and used between passages 3 and 5. Confluent HUVECs were resuspended and plated (3.5×10^5 cells/ml) in T75 flasks. Afterwards, cells were either grown to confluency again, exposed or otherwise to medium plus 30 mmol/l glucose and/or L-carnitine (2–10 and 30 mmol/l) or L-carnitine alone, so that after 96 h cells could be employed for apoptosis evaluation and AQP1 protein quantification.

Cytofluorimetric analysis of endothelial cell apoptosis was performed by annexin V-propidium iodide labeling. HUVECs were prepared for the annexin V-propidium iodide assay by combining floating and trypsin-released attached cells. Samples were centrifuged to pellet cells, washed thoroughly, resuspended in annexin binding buffer, and labeled with annexin V- fluorescein and propidium iodide as per the kit instructions (Bender Medsystem, Vienna, Austria, Europe). Samples were analyzed immediately by flow cytometry (BD Biosciences, FacsCalibur, Franklin Lakes, NJ).

AQP-1 western blot analysis was performed as previously described.[37]

L929 experimental protocol

A mouse fibroblast L929 cell line was grown as a monolayer in modified Eagle's medium containing nonessential aminoacids (1%) and supplemented with 10% fetal calf serum, and 50 μg/ml gentamycin. L929 were cultured by standard procedures.[24] The tests were performed in 96-well tissue culture plates (2000 cells/cm^2) in 200 μl/well of each test solution. The PD solutions employed were those used in our rat studies, and were diluted 1:1 (v/v) with modified Eagle's medium with or without L-carnitine (0.25–30 mmol/l). These PD solutions and controls (only modified Eagle's medium) were added to six parallel wells. After 3 days, the effects of L-carnitine on L929 viability and growth were assessed by the MTT method and [^{3}H]-thymidine incorporation test, respectively.

MTT assay

After cell stimulation as described above, a solution of MTT (0.5 mg/ml) was added to each well. After 3 h of incubation, cells were washed before addition of 200 μl dimethylsulfoxide. The plates were put in the dark for 30 min at 37 °C, and the spectrometric absorbance at 540 nm was read, using a microplate reader (SpectraMAX 190, Molecular Devices, San Jose, CA).

[^{3}H]-Thymidine incorporation assay

After cell stimulation as described above, [^{3}H]-thymidine (0.0185 MBq/well) was added for 4 h of incubation at 37 °C. Cells were then washed (FilterMate, 96 Samples Cell Harvester, Perkin

Elmer) and cell-incorporated radioactivity was measured in a scintillation counter (TopCount NXT, Scintillation and Luminescence Plate Counter, Perkin Elmer, Waltham, MA).

Statistical analysis

Results are presented as mean ± s.d. or as mean ± s.e.m., as indicated. For comparison of data, analysis of variance followed by Bonferroni's analysis or two-tailed Student's *t*-test were used (GraphPad Software, San Diego, CA). Significance was defined as a *P*-value <0.05.

DISCLOSURE

AA is an employee of CoreQuest. All the other authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Table S1. Complaints checked before, during, and after the 5 consecutive days of L-carnitine addition (5 g) to the nocturnal solution bag (1.5% glucose) in CAPD patients.
Table S2. Vital signs and electrocardiogram findings in CAPD patients treated with L-carnitine (5 g) added to the nocturnal solution bag (glucose 1.5%).
Table S3. Laboratory data before and after the 5 consecutive days of L-carnitine addition (5 g) to the nocturnal solution bag (1.5% glucose) in CAPD patients.
Table S4. Effects of L-carnitine addition (5 g) to the nocturnal solution bag (1.5% glucose) in CAPD patients.
Figure S1. PD transport parameters in C57 mice.
Figure S2. Transport of urea: effect of dialysate and AQP1 deletion.
Figure S3. Flow cytometry analysis of endothelial cell apoptosis measured by the annexin V-propidium iodide assay (A) and the MTT assay (B).
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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