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L-Carnitine improves cognitive and renal functions in a rat model of chronic kidney disease

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HIGHLIGHTS

- L-Carnitine improved creatinine and BUN levels in CKD rats.
- L-Carnitine treatment attenuated histological damage following CKD.
- CKD rats showed impaired cognition, which ameliorated by L-carnitine treatment.

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ABSTRACT

Over the past decade, the prevalence of chronic kidney disease (CKD) has reached epidemic proportions. The search for novel pharmacological treatment for CKD has become an area of intensive clinical research.
L-Carnitine, considered as the "gatekeeper" responsible for admitting long chain fatty acids into cell mitochondria. L-Carnitine synthesis and turnover are regulated mainly by the kidney and its levels inversely correlate with serum creatinine of normal subjects and CKD patients.
Previous studies showed that L-carnitine administration to elderly people is improving and preserving cognitive function. As yet, there are no clinical intervention studies that investigated the effect of L-carnitine administration on cognitive impairment evidenced in CKD patients.
Thus, we aimed to investigate the effects of L-carnitine treatment on renal function and on the cognitive performance in a rat model of progressive CKD.
To assess the role of L-carnitine on CKD condition, we estimated the renal function and cognitive abilities in a CKD rat model.
We found that all CKD animals exhibited renal function deterioration, as indicated by elevated serum creatinine, BUN, and ample histopathological abnormalities. L-Carnitine treatment of CKD rats significantly reduced serum creatinine and BUN, attenuated renal hypertrophy and decreased renal tissue damage.
In addition, in the two way shuttle avoidance learning, CKD animals showed cognitive impairment which recovered by the administration of L-carnitine.
We conclude that in a rat model of CKD, L-carnitine administration significantly improved cognitive and renal functions.

1. Introduction

The world's disease profile is nowadays rapidly changing and chronic life circumstances, including chronic kidney disease (CKD), account for a majority of global morbidity and mortality [1].

CKD encompasses a spectrum of different pathophysiological processes associated with abnormal kidney function and progressive decline in glomerular filtration rate. CKD is manifested by gradual

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reduction of nephron number and consequent deterioration of renal function. Renal responses to nephron number reduction are mediated by various vasoactive hormones, cytokines and growth factors [2]. Short-term adaptations to injury, such as renal hypertrophy and hyperfiltration, may become maladaptive with time progression. Furthermore, altered intra-renal microcirculation and increasing intra-renal pressure predispose to sclerosis and further dropout of the remaining nephrons [3,4]. Concomitant activation of intra-renal renin-angiotensin system is highly contributory to the initial adaptive hyperfiltration, the subsequent maladaptive hypertrophy and sclerosis, and initiation of intra-renal inflammatory processes [3].

The kidneys play an important role in L-carnitine metabolism, excretion, secretion and re-absorption [5]. L-Carnitine, the 3-hydroxy–trimethyl-aminobutyric acid, is a highly polar, low molecular weight compound secreted by all animal species and by most microorganisms and plants [6]. In animals, L-carnitine is synthesized almost exclusively in liver and kidneys and secondarily in the brain [7]. L-Carnitine has an obligatory role in β-oxidation of long chain fatty acids, it facilitates entry of long chain fatty acids into mitochondria for utilization in energy generating processes [6].

In CKD patients both plasma and muscle L-carnitine levels are elevated, presumably due to the compensatory activity of the liver [8,9]. In chronic hemodialysis patients, serum levels of free L-carnitine are subnormal, and rapidly decreasing up to 40% of the baseline, during dialysis sessions [10].

Previous studies showed that L-carnitine supplementation have some benefits in renal injury [11–15]. However, in contrast to these findings, Liu et al. found that L-carnitine may potentially disturb kidney function by altering renal protein levels of rat organic ion transporters [16]. The pathophysiological processes associated with abnormal kidney function include cognitive impairments. The severity of CKD correlates with the cognitive impairment, independent of age, education and other variables [17]. The prevalence of global cognitive impairment in CKD patients is more than twice that of the age-matched general population, and it was not explained by commonly measured metabolic alterations associated with CKD [18]. A meta-analysis examining the effects of L-carnitine in mild cognitive impairment and mild (early) Alzheimer's disease showed beneficial effects on both clinical scales and psychometric tests [19]. Malaguarnera et al. showed that L-carnitine administration to centenarians improved cognitive function assessed by the Mini-Mental State Examination [20].

Thus, in the present study, we aimed to investigate the effects of prolonged L-carnitine treatment on histopathological, biochemical and cognitive outcomes, in a CKD rat model.

2. Materials and methods

2.1. Animals

Thirty-one Wistar male rats, weighing 350–400 g, were purchased from Harlan Laboratories and were given 7 days of acclimation in the institutional animal housing facility. Rats were housed four per cage (30L × 30W × 18H cm). Room temperature maintained at 23 ± 1 °C with 67% humidity at 12:12 day/night cycle (lights on at 06:00). Food and water access allowed ad-libitum. This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All efforts were made to minimize animal suffering.

2.2. Surgery procedure

Animals were randomly subjected to 5/6 nephrectomy conducted in a single surgery procedure, comprised of right unilateral nephrectomy and ligation of 2/3 of the left kidney, without excision of the ligated tissue, or were subjected to sham operation and served as controls.

All operations were carried out under 90 mg/kg ketamine and 10 mg/kg xylazine (i.p).

Prior to the anesthesia, s.c rymadil [50 mg/ml] 0.1 cm^3 with 0.9 cm^3 physiological saline 0.9%/rat was injected for pain relief the rats received.

Throughout the entire recovery period (14 days), rats were individually inhabited in a heated room (25–27 °C; ~67% humidity), to avoid hypothermia or other complications. During the first 3 days of post-operation recovery period, all rats received twice a day subcutaneous rymadil injections (5 mg/100 g body weight).

Following the 14-days recovery period, animals were randomly assigned into two treatment conditions: treatment with saline (sham; $n = 7$, CKD; $n = 9$) or L-carnitine (Sigma-Aldrich, St Louis, MO, USA) 250 mg/kg body weight (sham-car; $n = 7$, CKD-car; $n = 8$) by daily intraperitoneal injections, for 8 consecutive weeks.

2.3. Animal sacrifice: blood and kidney sampling

Following 8 weeks of treatment, all rats were sacrificed. Blood samples were procured from abdominal aorta, left to clot for 1 h at room temperature and centrifuged for 20 min at 2000 × *g*. Serum samples were collected and stored at −80 °C until assayed.

Left kidney was excised at sacrifice, weighed, preserved in 4% formalin, and subsequently embedded in paraffin. Large sections (1 μm) were cut perpendicularly to renal capsule, in order to ensure that both cortex and medulla are presented in each section. Later, from four animals from each group, Paraffin-embedded slides were prepared by a standard procedure and stained with hematoxylin-eosin, for histopathologic examination under a light microscope. All renal tissues slides were blindly examined by pathologist and senior nephrologist, for tubular and glomerular injury and extracellular matrix deposition. Hyaline area was measured by computerized program for morphologic analysis (CMS-2-M).

2.4. Serum creatinine and BUN measurement

Serum creatinine was assessed by buffered kinetic Jaffé reaction. Serum Blood Urea Nitrogen (BUN) was estimated by urease and glutamate dehydrogenase-based kinetic assay. Both analyses were performed on Cobas-Mira autoanalyser (Hoffmann-LaRoche, Switzerland).

2.5. Two way shuttle avoidance learning

Two way shuttle avoidance test was carried out to assess the effects of CKD procedure and L-carnitine treatment on cognitive-related behavior.

The two-way shuttle avoidance box (60 cm × 26 cm × 28 cm) is divided by an opaque partition with a small (10 cm × 8 cm) passage door into two equal square compartments (A and B). Rats were placed individually in the test chamber, to which they acclimated for 5 min. Each rat was tested in a 60 trials session, comprised of conditioned stimulus (80 dB, 10 s tone) that followed by an unconditioned stimulus (10 s, 0.8 mA foot shock), with an inter-trial-interval of 1 min. Performance was scored as avoidance (shuttling to the adjacent compartment while the tone is on), escape (shuttling to the adjacent compartment during shock application) or freezing (no shuttling to the other compartment or shuttling to the adjacent compartment after the completion of the shock). The shuttle-box (Campden, UK) was connected to a computer, the onset latencies are measured automatically by Kinder Scientific software that allows the delivery of electrical shocks in a timed controlled manner and scored avoidance, escape and freezing responses.

2.6. Open field test

The open field is made of a black lusterless Perspex box (100L × 100W × 40H cm) placed in a dimly lit room (50 lx). The rat was placed in the corner of the open field (facing the wall) and given 5 min of free exploration. The behavior was videotaped by a CC TV Panasonic camera and analyzed off-line with Ethovision XT software (Noldus, The Netherlands) for activity and freezing duration.

2.7. Statistical analysis

The results are presented as means ± standard error of the means (SEM). Two-way ANOVA was applied to evaluate statistical differences between the conditions (sham vs. CKD) and treatment (saline vs. Car), followed by a Post-hoc t-tests. Differences yielding P value less than 0.05 were considered statistically significant.

3. Results

3.1. L-Carnitine treatment significantly decreased the weight gain of the CKD remnant kidney

Kidney weight was determined in all experimental groups eight weeks after surgery, as an indication of post-nephrectomy renal hypertrophy and/or compensatory growth (Fig. 1). We found a significant effect for CKD [$F(1,27) = 7.63$, $P < 0.01$], for L-carnitine treatment [$F(1,27) = 7.75$, $P < 0.01$] and for CKD X L-carnitine treatment interaction [$F(1,27) = 28.1$, $P < 0.0001$]. Expectantly, CKD group demonstrated a significant weight gain compared to the sham-operated [$t(14) = 5.15$, $P < 0.0001$]. L-Carnitine treatment significantly decreased the weight gain observed in the CKD group [$t(15) = 3.78$, $P < 0.002$]. No statistical significant difference was found between the sham and sham-car groups. Finally, there was a significant difference between the sham and CKD-car groups [$t(13) = 5.26$, $P < 0.0001$].

3.2. L-Carnitine treatment significantly improved kidney functioning and attenuated histological damage in CKD rats

Examining serum creatinine (Fig. 2A), we found a significant effect for CKD [$F(1,27) = 55.88$, $P < 0.0001$], for L-carnitine treatment [$F(1,27) = 7.35$, $P < 0.011$], and for CKD X L-carnitine treatment interaction [$F(1,27) = 4.57$, $P < 0.042$]. Specifically, the CKD group showed more than two-folds increase of creatinine compared with the sham-operated controls [$t(8) = 6.43$, $P < 0.0001$]. L-Carnitine treatment effectively and significantly reduced this augmentation [$t(15) = 2.75$, $P < 0.015$]. Yet, there was a significant difference between the sham and CKD-car groups [$t(7) = 5.43$, $P < 0.001$].

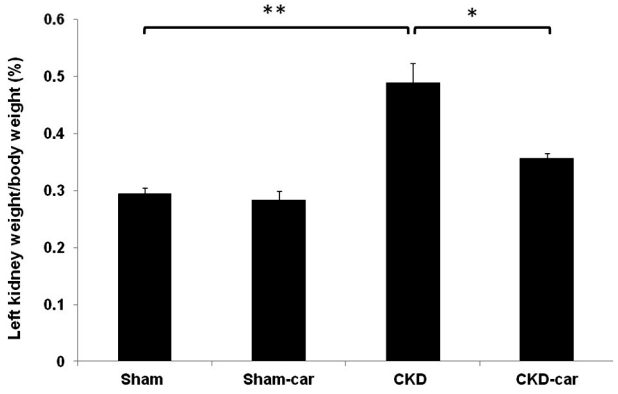

Fig. 1. The CKD rats showed higher left kidney weight compared with the Sham and Sham-car groups. CKD-car group showed recovery of the remnant left kidney weight; *$P < 0.002$, **$P < 0.0001$.

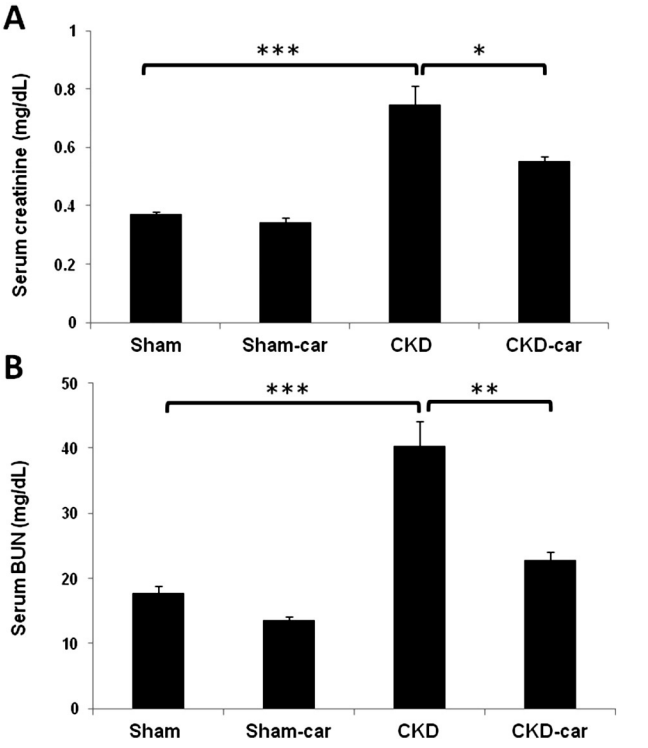

Fig. 2. CKD rats showed a significant elevation of serum creatinine (A) and BUN (B) levels compared with the Sham and Sham-car groups. CKD-car group showed a significant reverse of both creatinine and BUN levels, compared with CKD group; *$P < 0.015$, **$P < 0.001$, ***$P < 0.0001$.

Examining BUN (Fig. 2B), we found a significant effect for CKD [$F(1,27) = 48.02$, $P < 0.0001$], for L-carnitine treatment [$F(1,27) = 21.98$, $P < 0.0001$], and for CKD X L-carnitine treatment interaction [$F(1,27) = 8.35$, $P < 0.008$]. Specifically, the CKD group showed more than two-folds increase of creatinine compared with the sham-operated controls [$t(9) = 5.94$, $P < 0.0001$]. L-Carnitine treatment effectively and significantly reduced this augmentation [$t(9) = 4.54$, $P < 0.001$]. Yet, there was a significant difference between the sham and CKD-car groups [$t(13) = 3.45$, $P < 0.004$].

Sham rats kidneys showed normal renal cortex histology irrespective of L-carnitine treatment (Fig. 3A and B). CKD rats showed collapse of glomerular tuft, adhesion to Bowman's capsule and interstitial inflammation (Fig. 3C). In contrast, in the CKD-car group L-carnitine treatment showed ameliorated signs of glomerular damage (Fig. 3D).

Similar to the renal cortex, sham rats showed normal tubular histology, irrespective of L-carnitine treatment (Fig. 4A and B). Kidneys of CKD rats demonstrated signs of profound tubular injury (Fig. 4C). Most of the tubule was dilated, epithelial nuclei were enlarged with narrow cytoplasm, and with detached tubular epithelium. Epithelial cells sloughing into the renal tubular luminae were caught up within the mucoprotein matrix. Patchy tubular necrosis and cast formation were observed in the tubules of the CKD rats. However, tubules of CKD-car rats showed histology close to that of normal controls; no signs of extracellular matrix deposition and epithelial cell sloughing (Fig. 4D).

In addition, CKD rats demonstrated hyaline casts (Fig. 5A), while CKD-car rats demonstrated no sign of hyaline casts formation. Percentage of hyaline-covered area per total area within the hypertrophic zone of the remnant left kidney was profoundly decreased in CKD-car group compared with that of the untreated CKD group (Fig. 5B).

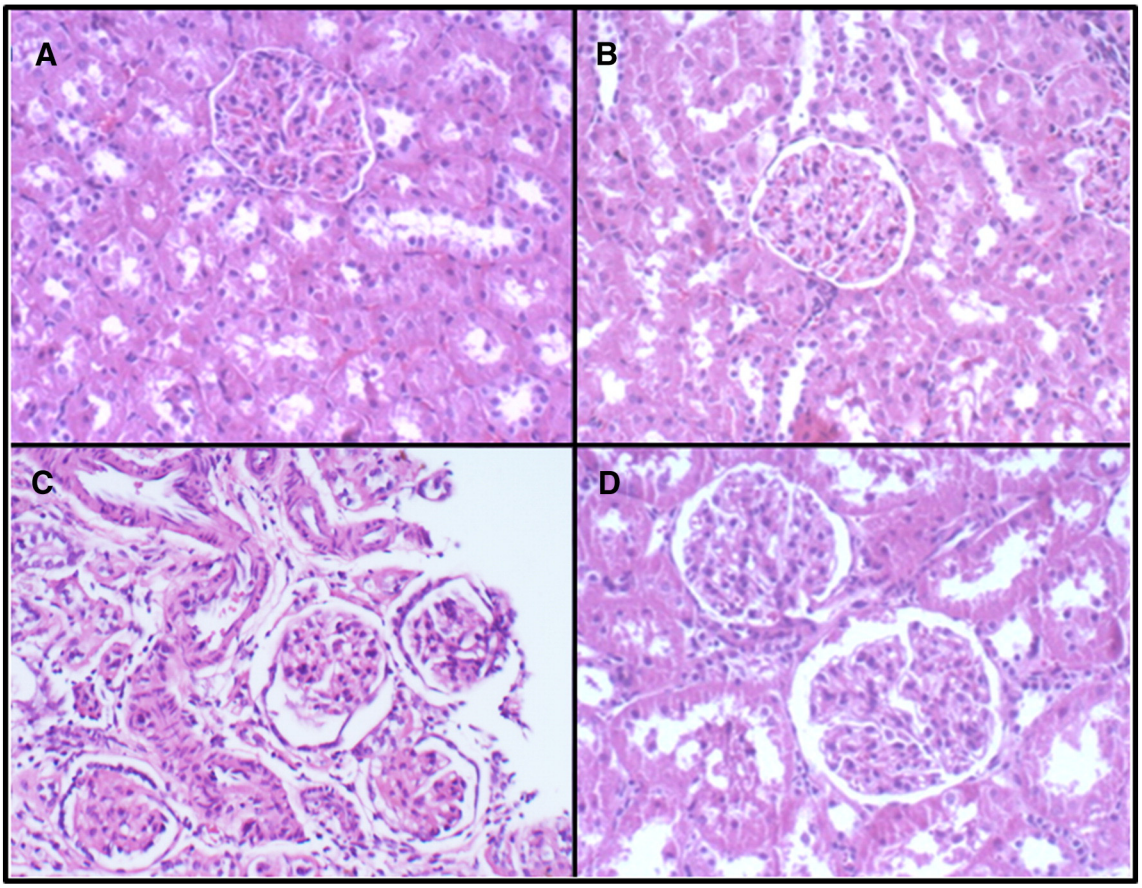

Fig. 3. Histopathological examination of renal glomerular tissue. Light microscopy images of hematoxylin-eosin (H&E) staining, paraffin embedded sections from kidney of (a) Sham, (b) Sham-car, (c) CKD and (d) CKD-car rats. Glomerular tuft area of the CKD group was shrunken with wrinkling of glomerular basement membranes, accompanied by reduction of capillary lumen diameter. In CKD-car group, histology of the glomeruli was similar to that of Sham groups (Magnification ×200).

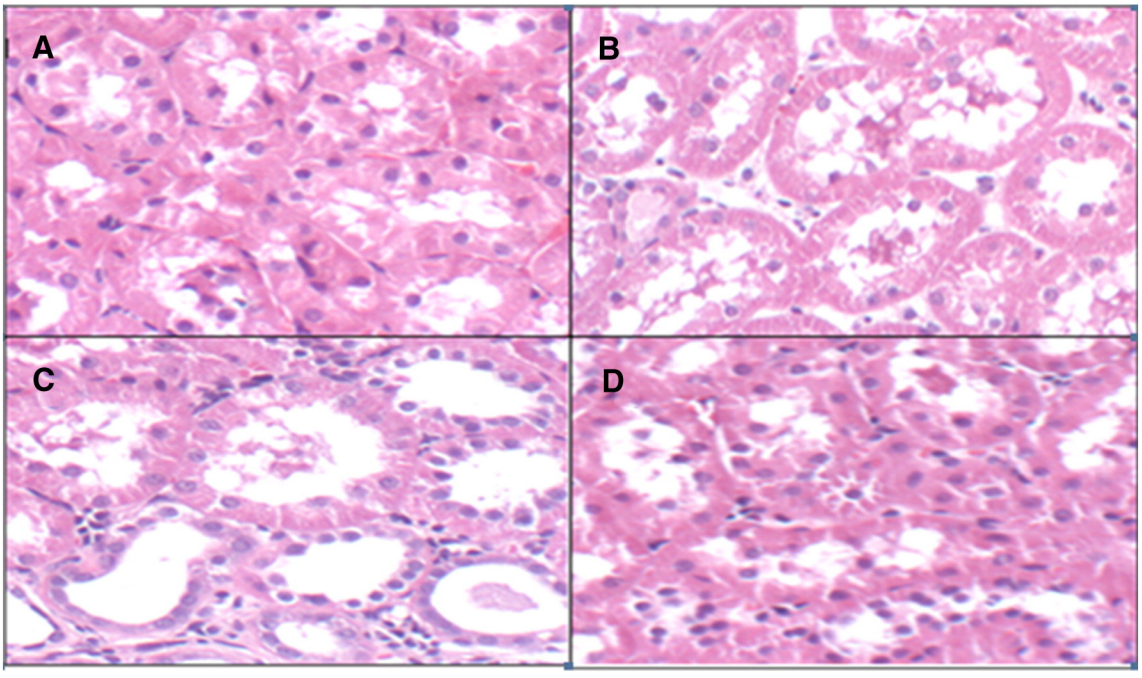

Fig. 4. Histopathological examination of renal tubular tissue. Renal tubules in H&E-staining, paraffin embedded sections from kidney of (a) Sham, (b) Sham-car, (c) CKD and (d) CKD-car rats. Patchy tubular necrosis and cast formation was observed in the tubules of the CKD rats. CKD-car group showed histology similar to that of Sham groups (Magnification ×200).

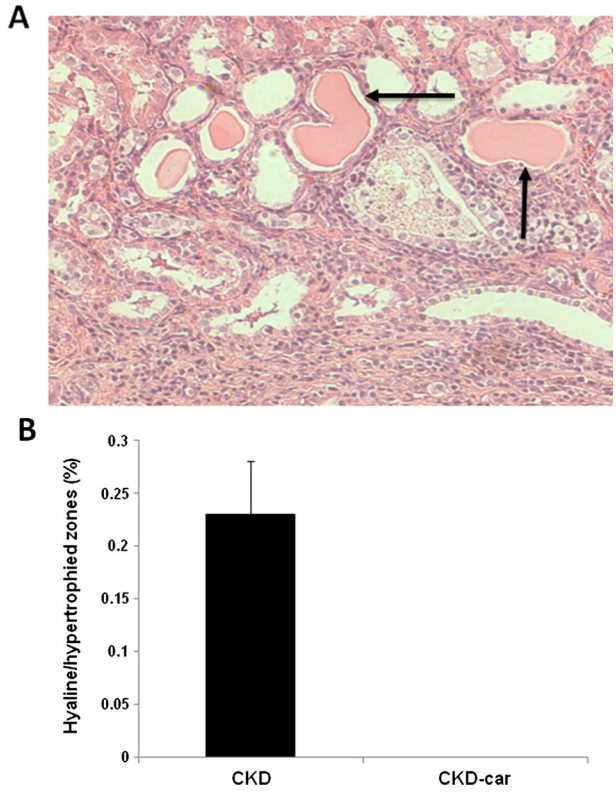

Fig. 5. Hyaline deposition in the CKD kidney distal tubule area. (A) H&E staining from kidney of CKD rats (Magnification ×200). Hyaline increments are marked by arrows. (B) Percentage of hyaline-covered area*(total area)$^{-1}$ in the hypertrophic zone of the remnant left kidney of the CKD and CKD-car groups. Hyaline-covered area in CKD group is rather extensive, while in the CKD-car there is no hyaline-covered areas ($P < 0.0001$).

3.3. L-Carnitine treatment significantly improved cognitive functioning in CKD rats

In the two way avoidance task we have measured a gradient of 3 coping behaviors: avoidance, escape and freezing (Fig. 6A). In a two-way ANOVA we examined the effects of condition (sham-operated vs. CKD) and treatment (saline vs. L-carnitine) on cognitive performance. Measuring the most adaptive behavior (i.e. avoidance), we found a significant main effect for condition [$F(1,24) = 118.5$, $P < 0.0001$] and for treatment [$F(1,24) = 20.28$, $P < 0.018$]. However, the condition X treatment interaction was found to be insignificant. Specifically, while CKD rats showed impaired avoidance behavior compared with the sham group, the treatment with L-carnitine (CKD-car group) recovered this decrement.

Examining the rate of escape behavior, we found no significant main effect for condition. However, we found significant effect for treatment [$F(1,24) = 10.47$, $P < 0.004$], and for the condition × treatment interaction [$F(1,24) = 66.91$, $P < 0.0001$]. Similar to the avoidance behavior, measuring escape behavior we found a decrease in the CKD group and that the treatment with L-carnitine recovered this decrease into a higher level, compared with the sham group.

Finally, examining the less adaptive behavior (i.e. Freezing), we found a significant main effect for condition [$F(1,24) = 138.53$, $P < 0.0001$] and insignificant effect for treatment. Complementarily to the significant decrease in both avoidance and escape behaviors observed in the CKD group, the latter showed a remarkable high rate of the maladaptive behavior, i.e. freezing. Treatment with L-carnitine recovered this increase, similar to that of the sham groups.

In order to exclude alternative effects due to general locomotor activity or anxiety-like behavior we measured in the open field test the locomotor activity (Fig. 6B) and freezing behavior (Fig. 6C) in all experimental groups. We found no differences between the groups.

4. Discussion

In the present study we conducted a CKD rat model comprised of 5/6 nephrectomy. Specifically, we conducted one kidney ablation and ligation of 2/3 of the contralateral kidney, in order to examine the role of L-carnitine on CKD consequences.

We found that the remnant kidney showed significant hypertrophied changes, followed by an increase of serum creatinine and BUN, and renal tissue damage. Together, these physiological changes validate our suggested CKD model. In the next step, we examined the effects of L-carnitine treatment on CKD deleterious consequences.

Indeed, the prolonged L-carnitine treatment effectively recovered the aforementioned adverse effects of CKD. Specifically, L-carnitine improved creatinine and BUN levels, ameliorated the severity of renal cortical proximal tubular necrosis and maintained greater renal function. In support of our findings, previous studies showed that L-carnitine ameliorates renal injury in rats [13–15].

Compensatory renal hypertrophy has been described many decades ago, as a whole set of changes in renal function and structure following reduction of functional kidney mass. Compensatory hypertrophy occurs in the remaining single kidney after contralateral nephrectomy [4], as a post-injury adaptation process enabling the animal to retain a great part of renal mass and function. At first, this process is on the account of the increased workload per nephron, and subsequently, on the account of compensatory gain of functional renal mass [3,4]. However, in contrast to unilateral kidney ablation, CKD leads to excessive hypercellularity of the remnant kidney accompanied by extracellular matrix deposition. Eventually it amplifies the development of renal tissue scarring and thus contributes to the progression of CKD [4]. Accordingly, our results showed that the treatment of CKD rats with L-carnitine prevented renal hypertrophy, ameliorated signs of glomerular damage, prevented signs of hyaline casts formation and signs of extracellular matrix deposition and epithelial cell sloughing. Together, the treatment with L-carnitine may be considered beneficial for the function of the remnant kidney.

Relating to quality of life, CKD patients suffer from cognitive impairment which correlates with the severity of CKD, independent of age, education and other variables [17,23]. Various human and animal models studies showed beneficial effects of L-carnitine treatment on cognitive function. Treatment with mixture of natural metabolites, including L-carnitine, reduced oxidative damage to murine brain and improved cognitive performance [24]. Patients with severe hepatic encephalopathy in a state of impaired cognitive function that were treated with Acetyl-L-carnitine showed significant cognitive improvement (e.g. logical memory, oral word association, judgment of line orientation) and a significant improvement in EEG [25]. In addition, clinical studies demonstrated that acetyl-L-carnitine provides cognitive and/or behavioral benefits in Alzheimer disease [19,26,27]. Thus, we aimed to examine whether our CKD rat model indeed leads to impaired cognitive functions and whether L-carnitine ameliorates these expected impairments. Utilizing a complex cognitive process manifested in the two-way avoidance task, we are able to depict a gradient of adaptive behaviors based on fear conditioning process [28]. The most adaptive behavior is to avoid foot-shock during the tone, regardless of the rat's position in compartment A or B (following the rule "no place is a safe place"). In the second level of adaptivity, the rat is not responding to the anteceding tone, therefore, getting the foot-shock and escaping to the other compartment from the one that it spent in while getting the shock. Finally, as the mal-adaptive behavior, the rat freezes during both the tone and the subsequent foot-shock and not avoiding/escaping into the alternative compartment. Our results showed that the CKD rats exhibited low level of both avoidance and escape behaviors and complementarily

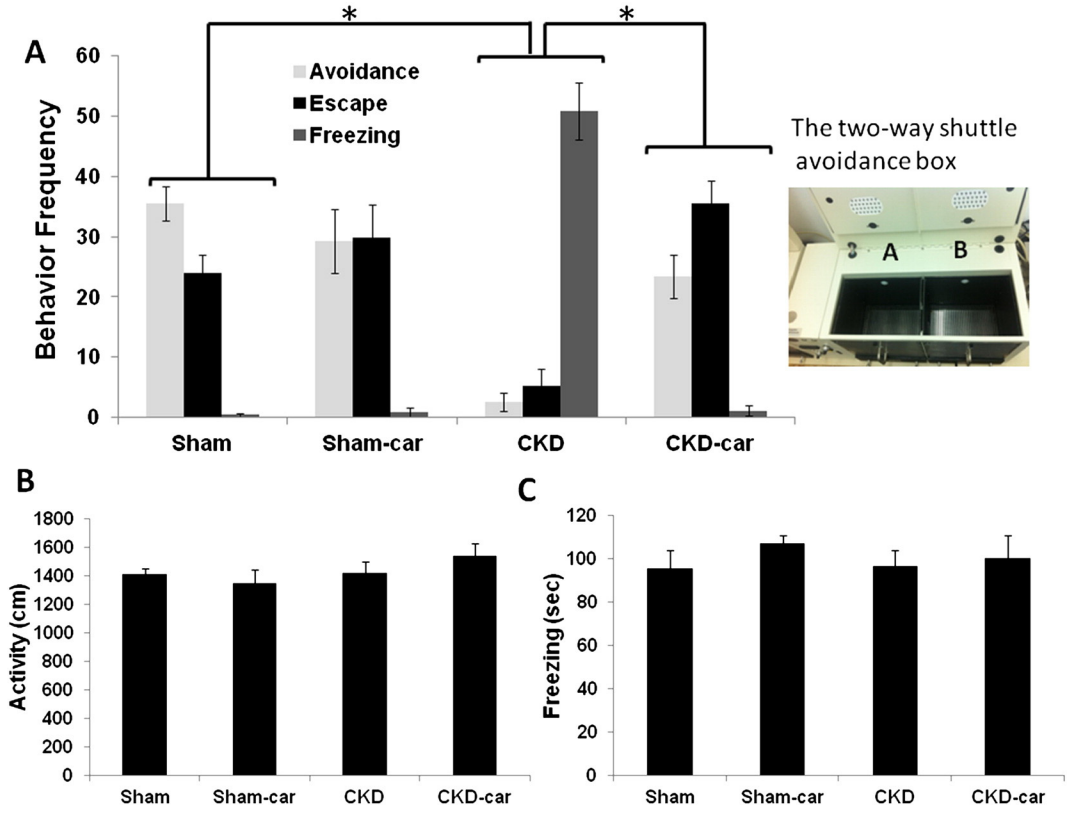

Fig. 6. (A) CKD group showed decreased rate of avoidance and escape behaviors and complementarily increased rate of freezing, compared with the sham group. However, treatment with L-carnitine recovered these impairments (*$P < 0.0001$). On the right panel, a photo of the two-way avoidance shuttle box comprised of compartments A and B, is presented. In the open field test we found no significant differences between the groups in locomotor activity (B) and freezing duration (C).

exhibited high level of the mal-adaptive behavior, i.e. freezing. L-Carnitine improved the deleterious effect of CKD and recovered the level of both avoidance and escape behaviors similar to that of the sham operated rats. Together, the CKD rats showed a decrease in learning ability which ameliorated by L-carnitine treatment. To further support the results of the two-way avoidance task we excluded the effects of general locomotor activity and anxiety-like behavior. To the best of our knowledge, we are the first to examine the effects of L-carnitine on cognitive function in CKD rat model.

Studies on possible mechanisms of L-carnitine-exerted organ protection suggest that L-carnitine acts as antioxidant and protect tissues from oxidative damage [29]. As we previously showed [30], oxidative stress impaired long term potentiating (LTP) as well as spatial learning and memory in the Morris water maze.

In addition, L-carnitine may reduce energy dissipation and provide optimal metabolic energy supplies according to physiological needs [31]. The beneficial effects of L-carnitine in Alzheimer's disease can also be explain by its ability to restore changes in membrane phospholipid metabolism found in subjects with Alzheimer's disease [19,32].

In conclusion, prolonged administration of L-carnitine exerted significant renoprotective effects in an experimental rat model of CKD, by slowing the deterioration of renal function, decreasing undesirable kidney hypertrophy and ameliorating histopathological hallmarks of renal tissue damage. A similar effect was found in the cognitive aspect; prolonged administration of L-carnitine recovered cognitive impairment observed in CKD condition.

Taken together, our results indicate that L-carnitine may be considered as a supportive treatment for CKD patients. Additional investigation is necessary to further scrutinize the specific mechanisms involved, as well as the clinical value of renal protection and cognitive consequences exerted by L-carnitine treatment.

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