Comparison of the Effects of L-Carnitine and Acetyl-L-Carnitine on Carnitine Levels, Ambulatory Activity, and Oxidative Stress Biomarkers in the Brain of Old Rats

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ABSTRACT: L-Carnitine and acetyl-L-carnitine (ALC) are both used to improve mitochondrial function. Although it has been argued that ALC is better than L-carnitine in absorption and activity, there has been no experiment to compare the two compounds at the same dose. In the present experiment, the effects of ALC and L-carnitine on the levels of free, acyl, and total L-carnitine in plasma and brain, rat ambulatory activity, and biomarkers of oxidative stress are investigated. Aged rats (23 months old) were given ALC or L-carnitine at 0.15% in drinking water for 4 weeks. L-Carnitine and ALC were similar in elevating carnitine levels in plasma and brain. Both increased ambulatory activity similarly. However, ALC decreased the lipid peroxidation (malondialdehyde, MDA) in the old rat brain, while L-carnitine did not. ALC decreased the extent of oxidized nucleotides (oxo8dG/oxo8G) immunostaining in the hippocampal CA1 and cortex, while L-carnitine did not. ALC decreased nitrotyrosine immunostaining in the hippocampal CA1 and white matter, while L-carnitine did not. In conclusion, ALC and L-carnitine were similar in increasing ambulatory activity in old rats and elevating carnitine levels in blood and brain. However, ALC was effective, unlike L-carnitine, in decreasing oxidative damage, including MDA, oxo8dG/oxo8G, and nitrotyrosine, in old rat brain. These data suggest that ALC may be a better dietary supplement than L-carnitine.

KEYWORDS: acetyl-L-carnitine (ALC); L-carnitine; ambulatory activity; malondialdehyde (MDA); nitrotyrosine; oxo8dG/oxo8G

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INTRODUCTION

L-Carnitine has been described as a conditionally essential nutrient for humans. L-Carnitine facilitates entry of long-chain fatty acids into mitochondria for utilization as fuel and facilitates removal from mitochondria of short-chain and medium-chain fatty acids that accumulate as a result of normal and abnormal metabolism.[1] Experimental data demonstrate an age-associated decrease of tissue levels of L-carnitine in animals, including humans, and an associated decrease in the integrity of the mitochondrial membrane.[2–4] Because carnitine is essential for translocation of fatty acids into mitochondrial matrix, it is plausible that carnitine deficiency would cause disorders in fatty acid utilization.[5] L-Carnitine deficiency is associated with cardiomyopathy,[6,7] and L-carnitine administration protects the myocardium against diphtheria toxin, ischemia, myocardial infarction, and adriamycin-induced damage.[8] ALC, like L-carnitine, is present in high concentration in the brain as well as muscle, and provides acetyl equivalents for the production of the neurotransmitter acetylcholine.[7,9,10] ALC has been shown to delay or reverse age-related deficits in mitochondrial function, such as in the heart and liver.[11–16] In addition, ALC improves age-associated cognitive dysfunction and neurodegeneration in animals[17–22] and in Alzheimer's patients,[23,24] as well as decreases oxidative stress.[20,25,26]

L-Carnitine and its acyl esters may act as an oxidant either having a primary antioxidant activity (inhibiting free radical generation, scavenging the initiating free radicals, and terminating the radical propagation reactions) or, more likely, functioning as a secondary antioxidant (repairing oxidized polyunsaturated fatty acids esterified in membrane phospholipids).[27,28] ALC inhibits lipid peroxidation and xanthine oxidase activity in rat skeletal muscle.[29] ALC reduces lipid peroxidation and lipofuscin concentration in aged rat brain.[30] ALC also inhibits oxidant-induced DNA single-strand breaks.[31] ALC may possess a direct antioxidant activity as demonstrated *in vivo*.[32,33] Related compounds, such as L-propionyl L-carnitine and L-carnitine, have been shown to have antioxidant activity by chelating metals,[34] and inhibiting the age-associated increase in lipid peroxidation.[25,26] An antioxidant role of L-propionyl L-carnitine has also been implicated in ischemia-reperfusion injury.[35] L-Carnitine in rats prevented doxorubicin cardiotoxicity as monitored by echocardiography, release of myosin light chain-1, and aldehydic lipid peroxidation products.[36] It was suggested that the protective effects of L-carnitine were due to its beneficial impact on energy metabolism and/or its antioxidant effects because of the attenuation of the doxorubicin-induced lipid peroxidation. ALC prevented oxygen radical–induced cell death in human diploid fibroblast cell lines, which was explained as due to increasing the activity of antioxidant enzymes and sustaining the activity of mitochondrial complex I–NADH ubiquinone reductase and complex IV–cytochrome oxidase of the electron transport chain.[37] In addition, ALC protected mitochondrial complex III ubiquinol cytochrome *c* reductase, perhaps as an iron chelator.[38]

Very little data are available concerning brain L-carnitine status of old rats. One report by Maccari *et al.*[4] showed that aged Sprague-Dawley rats (30 months) exhibited a statistically significant decrement of total L-carnitine levels in the brain, serum, heart, and tibial muscle, accompanied by a marked increment in the liver. Another report[39] demonstrated that L-carnitine levels in muscles of old male Wistar rats were significantly lower than that in young animals. In our previous dose-response study,[3] we demonstrated that ALC at a 0.15% level (in drinking water) can increase

plasma and brain carnitine levels as efficiently as 0.5% and 1.5%, but the highest dose (1.5%) causes oxidative damage.[16] ALC is more widely used than L-carnitine in animal research and clinical trials to gain metabolic benefits to the brain, heart, liver, and other organs. ALC, compared with L-carnitine, is more efficient to cross the blood-brain barrier.[40] There are limited data to compare the two compounds at the same dose in aging animals. The aim of the present study was to compare the effects of the relatively lower optimal dose (0.15%) of L-carnitine and ALC on the levels of free, acyl, and total L-carnitine in plasma and brain, ambulatory activity, and biomarkers of oxidative stress, including lipid peroxidation, protein oxidation, and oxidative nucleic acid damage in the brain of old rats.

MATERIALS AND METHODS

Materials

ALC (hydrochloride salt) was a gift of Sigma-Tau (Pomezia, Italy). All other reagents were reagent grade or the highest quality available from Sigma (St. Louis, MO), unless otherwise indicated.

Animals

Fischer 344 male rats were obtained from the National Institute on Aging. Control animals were fed an AIN93M diet from Dyets (Bethlehem, PA) and MilliQ (Millipore) water (pH 5.2). The rats in the experimental groups were fed drinking water with either 0.15% ALC or L-carnitine for 4 weeks. The young rats were 4.5 months and the old ones were 24.5 months at the start of the experiment; they were 5.5 months (young) and 25.5 months (old) when they were terminated. Prior to tissue collection, animals were fasted overnight, and sacrificed between 9:00 and 12:00 in the morning. The animals were fully anesthetized with ether prior to exsanguinations by perfusion with PBS. The brains were removed, immediately put into liquid nitrogen, and stored in a −80°C freezer until analysis.

Assay for Total L-Carnitine, Free L-Carnitine, and Acyl L-Carnitine

Levels of total L-carnitine, free L-carnitine, and acyl L-carnitine were determined with an enzymatic cycling method with L-carnitine dehydrogenase.[41,42] Briefly, brain tissues were homogenized with 5 volumes of ice-cold Tris-HCl buffer (pH 7.5) with 100 mM sodium chloride and 40 w/v% sucrose, and then deproteinized with ice-cold 15% trichloroacetate (480 μL/mL homogenate) and centrifuged. The pH of the resulting supernatants was neutralized with 1 M potassium hydroxide. After neutralization, specimens were again centrifuged and analyzed immediately with the L-carnitine assay kits (Asahi Chemical Industry, Tagatagun, Japan) as instructed.

Ambulatory Activity Tests

Rats were moved each night from group housing to individual cages (48 × 25 × 20 cm) at 4 h prior to the quantification of ambulatory parameters. The testing room

was on a 12-h light/dark cycle (lights on 6 A.M. to 6 P.M.). At 8 P.M., a very low light illuminated the test subjects for video tracking. Quantification began at 9 P.M. and continued for 4 h. One hour later, the low light was turned off and the room remained in total darkness until 6 A.M., when the standard light/dark cycle began. A video signal from a camera suspended directly above the individual cages was fed directly into a Videomex-V (Columbus Instruments, Columbus, OH) computer system running the Multiple Objects Multiple Zones software. The system quantified ambulatory activity parameters and was calibrated to report distance traveled in centimeters. In addition to total distance traveled, the time each subject spent in ambulatory (locomotor), stereotypic (grooming), and resting (nonmovement) activity was recorded by an IBM computer. At 9 A.M., animals were removed from individual housing and returned to group housing. Results are shown as the mean centimeters traveled per hour ± SEM.[16,43]

Malondialdehyde Assay

Lipid peroxidation was assayed using a sensitive and specific gas chromatography–mass spectrometry method for detecting malondialdehyde (MDA).[44–46] The GC-MS assay was carried out as reported previously with a Hewlett-Packard 5890 Series II gas chromatograph interfaced to a 5989 mass spectrometer equipped with a J&W Scientific (Folsom, CA) DBWAX capillary column (15 mm × 0.25 mm i.d., 0.25-μm film thickness). Briefly, the protein-bound MDA is hydrolyzed with H_2SO_4, MDA is converted into a stable derivative using pentafluorophenyl hydrazine at room temperature, and the derivative is detected using the GC-MS in the negative chemical ionization mode. 2,6-*tert*-Butyl-4-methylphenol (BHT, 67 μmol) was added at the beginning of the assay to prevent artifactual *in vitro* oxidation.

Immunohistochemical Studies on Nitrotyrosine and Oxo8dG/Oxo8G

Subsets of rats from each treatment condition ($n = 4$ young rats; $n = 5$ old rats; $n = 3$ old rats treated with 0.15% ALC; $n = 3$ old rats treated with 0.15% L-carnitine) were anesthetized with ether and perfused with 4% paraformaldehyde for 1.5–2 h. The brain was removed and postfixed for preparing paraffin sections. Sections of hippocampus were incubated with polyclonal antinitrotyrosine (1:100, Upstate Biotechnology, NY) monoclonal anti-8-hydroxy-2′-deoxyguanosine/8-hydroxyguanosine (oxo8dG/oxo8G; 1:2000; QED Bioscience, San Diego, CA) and visualized by using previously published immunocytochemical methods.[21] Two independent analyses were done on each rat. To quantify the extent of oxo8dG/oxo8G immunolabeling, a 525 × 410 μm area of staining was captured by using a 2.5 photo eyepiece, a Sony (Tokyo) high-resolution charge-coupled device (CCD) video camera (XC-77), and the built-in video capture capabilities of a Macintosh 8100/80AV. All sections from a given region were captured sequentially during one session and were analyzed blind with respect to treatment condition. Subsequently, public domain image analysis software (IMAGE 1.55, NIH) and gray-scale thresholding were used to separate positive staining from background and to calculate the percentage of area occupied by oxo8dG/oxo8G immunoreactivity.

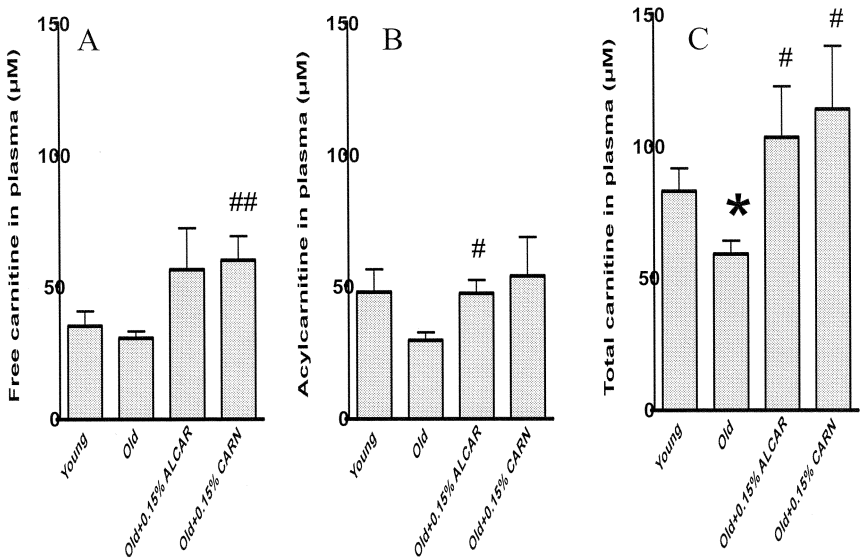


FIGURE 1. Free **(A)**, acyl **(B)**, and total **(C)** carnitine levels in plasma of young, old, and old rats treated with 0.15% ALCAR or L-carnitine (CARN) in drinking water for 4 weeks. $^{*}P < 0.05$ vs. young; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. old.

RESULTS

Plasma L-Carnitine Levels

The results from rat plasma are shown in FIGURE 1 (A: free L-carnitine; B: acyl carnitine; C: total L-carnitine). There is an age-related decrease of acyl L-carnitine and total L-carnitine in plasma, although not free L-carnitine. L-Carnitine and ALC elevated carnitine levels in plasma in old rats similarly (FIG. 1).

Brain L-Carnitine Levels

The results from rat brain are shown in FIGURE 2 (A: free L-carnitine; B: acyl carnitine; C: total L-carnitine). There is an age-related decline in free and total L-carnitine levels in the brain. L-Carnitine and ALC elevated carnitine levels in the brain in old rats similarly (FIG. 2).

Ambulatory Activity

Ambulatory activity declined markedly with age. This decline was partially reversed by both ALC and L-carnitine treatment (FIG. 3). L-Carnitine and ALC had a similar effect on increasing ambulatory activity in old rats.

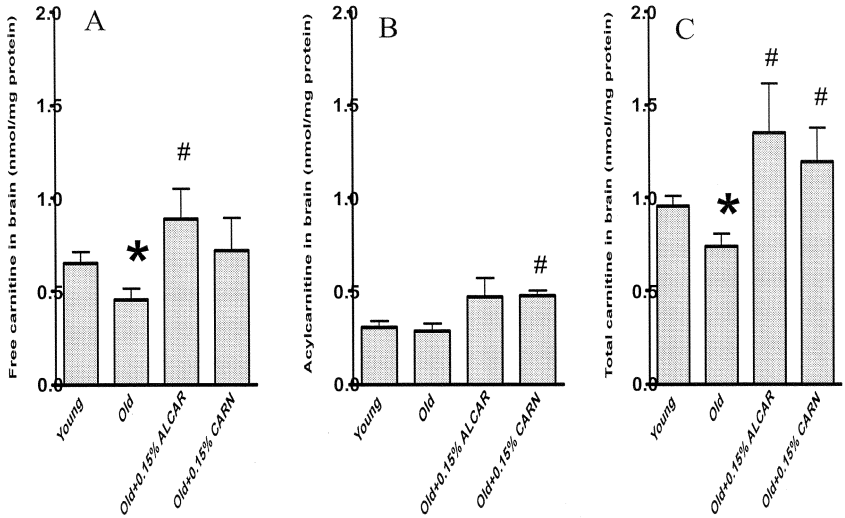


FIGURE 2. Free **(A)**, acyl **(B)**, and total **(C)** carnitine levels in brain of young, old, and old rats treated with 0.15% ALCAR or L-carnitine (CARN) in drinking water for 4 weeks. $^{*}P < 0.05$ vs. young; $^{\#}P < 0.05$ vs. old.

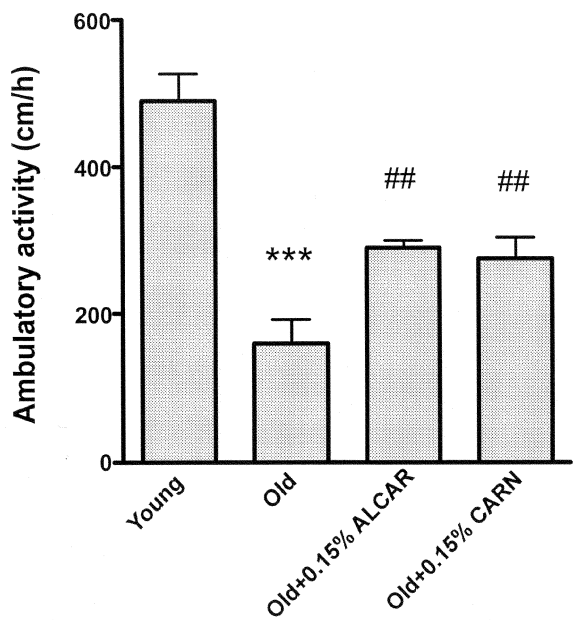


FIGURE 3. Ambulatory activity in young, old, and old rats treated with 0.15% ALCAR or L-carnitine (CARN) in drinking water for 4 weeks. $^{***}P < 0.001$ vs. young; $^{\#\#}P < 0.01$ vs. old.

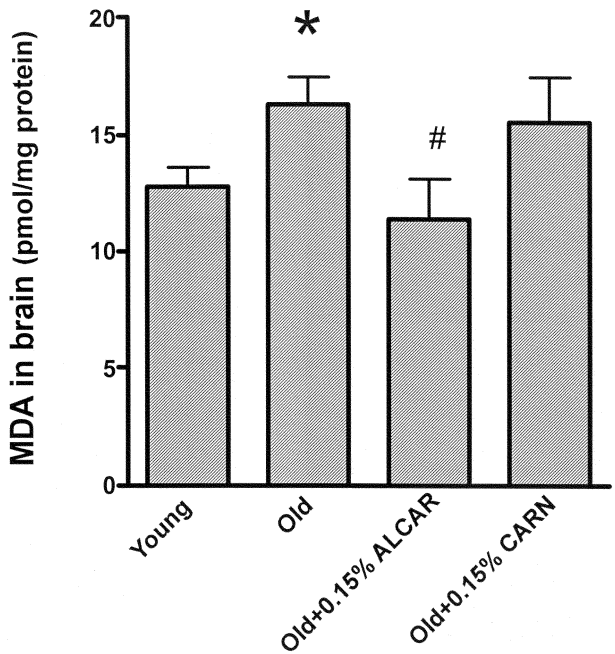


FIGURE 4. Malondialdehyde (MDA) in young, old, and old rats treated with 0.15% ALCAR or L-carnitine (CARN) in drinking water for 4 weeks. $^{*}P < 0.05$ vs. young; $^{\#}P < 0.05$ vs. old.

Lipid Peroxidation

As shown in FIGURE 4, old rats had a significant increase in MDA in their brain when compared to young rats. ALC decreased the MDA level in the old rat brain, but L-carnitine did not (FIG. 4).

Oxo8dG/Oxo8G Immunostaining of Young and Old Brain: Effects of ALC and L-Carnitine Feeding

We showed previously that the oxidative damage to nucleic acids stained by oxo8dG/oxo8G immunoreactivity occurred predominantly in RNA (>90%).[21] Hippocampal area CA1, underlying white matter, and frontoparietal cortex showed an effect of age [$F(1,6) = 8.82$, $P < 0.025$] on levels of oxo8dG/oxo8G immunoreactivity (by repeated ANOVA analysis). Significant increases in oxo8dG in the deep layers and white matter of the frontoparietal cortex along with increases in CA1 and CA4 account for the majority of the age effects (FIG. 5). To compare differences in 0.15% ALC with 0.15% L-carnitine for each brain region, repeated-measure ANOVA was used. Overall, ALC resulted in significantly reduced oxo8dG, which post hoc comparisons (Scheffe) showed to be significant in the frontotemporal cortex ($P < 0.019$) and in area CA1 ($P < 0.001$). The level of oxo8dG/oxo8G immunoreactivity was not reduced in rats treated with L-carnitine as compared to untreated controls.

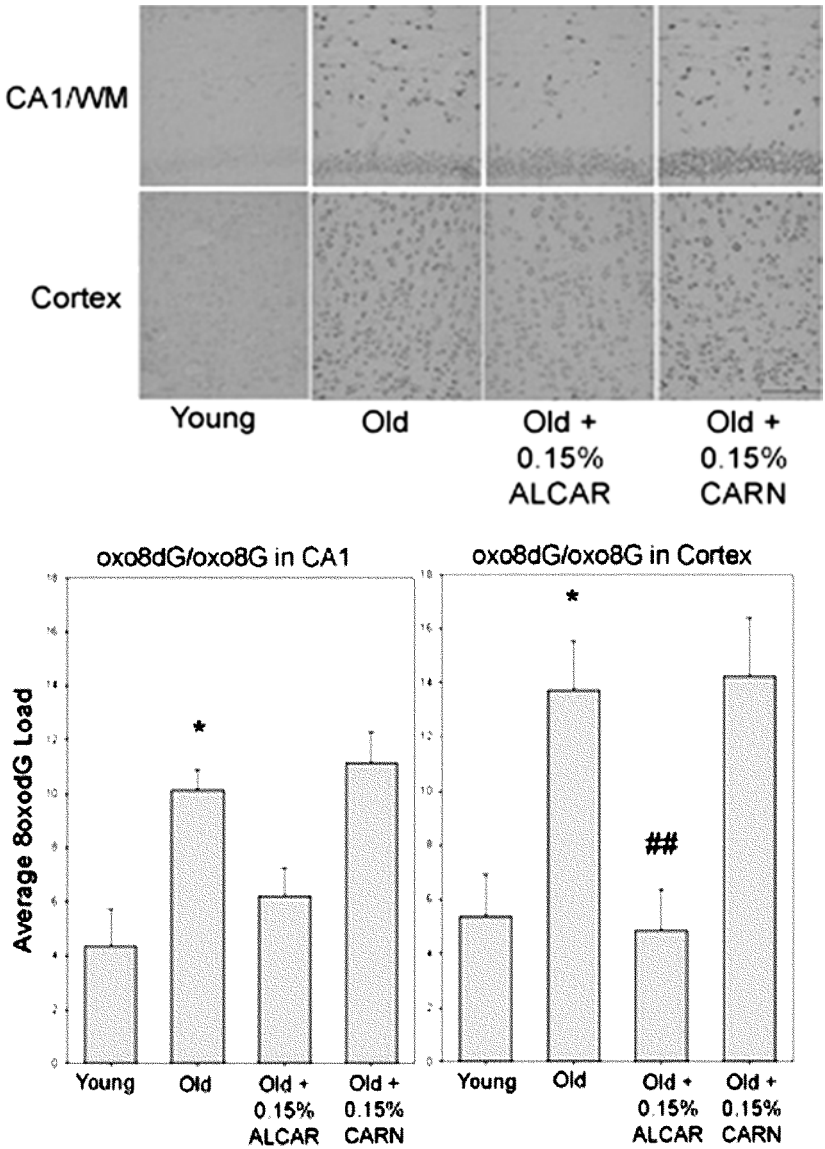
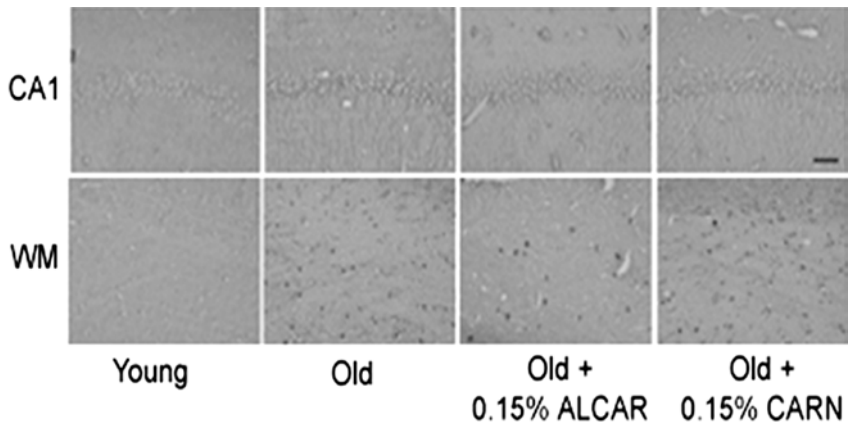


FIGURE 5. (Top) Representative photographs for oxo8dG/oxo8G immunostaining in brain area CA1, cortical neurons of the frontoparietal cortex, and underlying white matter. (Bar = 50 μm.) **(Bottom)** Average nitrotyrosine immunostaining rankings in area of hippocampal CA1 and cortical neurons of the frontoparietal cortex. Old rats, compared to young rats, showed significantly higher oxo8dG/oxo8G immunoreactivity in hippocampal CA1 and cortical neurons of the frontoparietal cortex. ALCAR reduced the extent of oxo8dG/oxo8G immunoreactivity in area CA1 and in neurons of the frontoparietal cortex in old rats, although only significant in the cortex. Old rats treated with 0.15% L-carnitine showed no reduction in age-associated oxo8dG/oxo8G immunoreactivity.



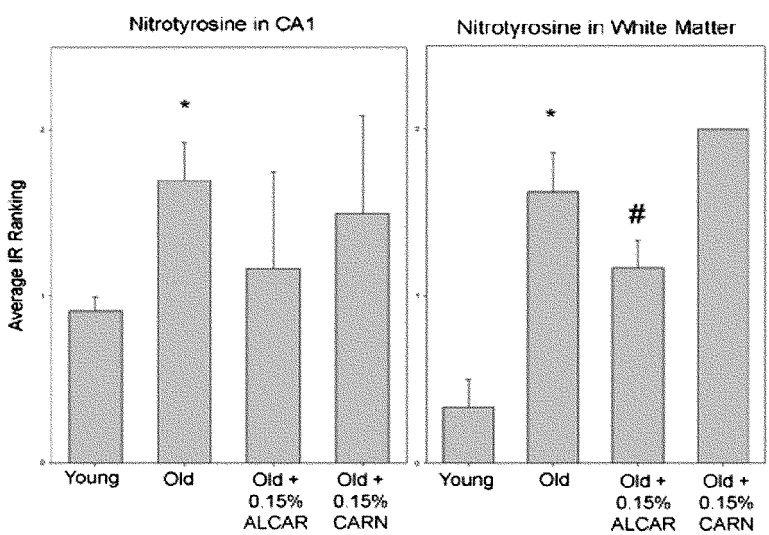


FIGURE 6. (Top) Representative photographs for nitrotyrosine immunostaining in brain area CA1 and white matter of the frontoparietal cortex. (Bar = 50 μm.) **(Bottom)** Average nitrotyrosine immunostaining rankings in area CA1 and in the white matter of the frontoparietal cortex. Old rats, compared to young rats, showed higher nitrotyrosine immunoreactivity in the hippocampal CA1 area and the white matter of the frontoparietal cortex. ALCAR significantly reduced age-associated increases in nitrotyrosine in the white matter, but L-carnitine did not.

Nitrotyrosine Immunostaining of Young and Old Brain: Effects of ALC and L-Carnitine Feeding

Old rats showed increased nitrotyrosine immunoreactivity relative to young rats in 3/4 regions of the hippocampus that included area CA4 ($P < 0.01$), CA3 ($P < 0.01$), and CA1 ($P < 0.03$) (Mann-Whitney U tests). Nitrotyrosine immunoreactivity in the dentate gyrus was not significantly different between young and old rats. In addition, there was a trend towards increasing nitrotyrosine levels in the white matter of cortex of old rats ($P < 0.03$), but levels in deep-layer cortical neurons of the frontoparietal cortex were similar in young and old animals. As shown in FIGURE 6, ALC and L-carnitine administration resulted in similar levels of nitrotyrosine immunoreactivity, except in the white matter where ALC significantly reduced staining, but L-carnitine did not ($P < 0.034$) (Kruskal-Wallis nonparametric test).

DISCUSSION

The current study directly compared the similar doses of ALC and L-carnitine in aged rats on measures of ambulatory activity; free, acyl, and total carnitine levels; and biomarkers of brain oxidative damage. Both compounds increased peripheral and central carnitine levels, and increased ambulatory activity in old animals. However, only ALC treatment resulted in a decrease in biomarkers of oxidative damage, including lipid peroxidation (MDA), protein oxidation (nitrotyrosine), and oxidative nucleic acid damage (oxo8dG/oxo8G) in the brain of old rats. The concentrations used here for L-carnitine and ALC are weight/volume. The corresponding mole concentrations for the 0.15% solution are 7.78 mM for L-carnitine and 6.25 mM for ALC, respectively. Therefore, the effect of ALC should be even greater if compared to the same mole concentration of L-carnitine.

There are difficulties with several enzymatic assays for L-carnitine, including a radiochemical method with L-carnitine *O*-acetyltransferase, and spectrophotometric methods involving L-carnitine acetyltransferase or L-carnitine dehydrogenase.[42] These methods measure NADH production, which has low molar absorptivity at 340 nm, making it difficult to assay carnitine in samples from carnitine-deficient diseases. In addition, the NADH produced is consumed by another dehydrogenase, which thus causes erroneous measurements. We have used a carnitine kit (Asahi Industrial, Tokyo), which is highly sensitive and specific for determining L-carnitine levels by use of carnitine dehydrogenase (EC 1.1.1.108, purified from *Alcaligenes* sp.). The method involves a new enzymatic cycling technique with NADH, thio-NAD^+, and L-carnitine dehydrogenase, and measures the increase of absorbance at 405 nm of thio-NADH produced during the reaction.[42] Due to the high sensitivity, this method allowed us to determine carnitine concentrations at a few micromoles per liter, or in less than 5 μL of plasma. Our results for plasma and brain tissues in young and aged Fischer 344 rats are consistent with those reported by Maccari *et al.*[4] in Sprague-Dawley rats, suggesting the method is quite reliable in measuring plasma and tissue carnitine levels. At the dose of 0.15%, ALC and L-carnitine were both effective in restoring the age-associated decrease of levels of plasma and brain carnitine to those of young rats.

Administration of 0.1% and 1.0% L-carnitine can significantly increase carnitine levels in young rat serum, liver, kidney, heart, and skeletal muscle.[47] A recent study showed that a daily dose of 100 mg/kg body weight ALC in drinking water elevated total carnitine levels in plasma, heart, skeletal muscle, and cerebral cortex in old rats.[48] Kuratsune *et al*. showed that patients with chronic fatigue syndrome have a deficiency of serum acyl carnitine, and ALC supplementation improves the daily activity and reduces the symptoms.[42,49] They investigated the brain uptake in rhesus monkeys of acetyl carnitine labeled in different positions by positron emission tomography and found a high uptake of [$^{2-11}$C]-acetyl-L-carnitine into the brain, suggesting that endogenous serum acetyl-L-carnitine has some role in conveying an acetyl moiety into the brain, especially under an energy crisis.[50] Oral and intravenous administration of multiple doses of ALC can increase its plasma and CSF concentration in patients suffering from Alzheimer's disease.[51] Consistent with our results, all of these results suggest that dietary supplementation of L-carnitine and ALC can elevate peripheral and central carnitine levels similarly.

Our previous report[16] showed that old rats had a remarkable decline in ambulatory activity and that this decline reflected a true loss in activity because the calculated speeds of animals, when they moved, were not significantly different from those of young rats. The results here show that ALC and L-carnitine administration increased the age-associated decline in ambulatory activity similarly.

Our results on MDA, nitrotyrosine, and oxo8dG/oxo8G (mainly RNA oxidation) in young and old rats are consistent with other reports and support the proposition that accumulated oxidative damage to macromolecules such as lipid, protein, and nucleic acid may be a major contributor to cellular aging and the degenerative diseases that accompany aging.[21,43,52–60] In a previous report,[16] we showed that, although 1.5% ALC significantly restored age-associated mitochondrial decay and ambulatory decline in old rats, the ALC administration caused an increase in oxidant production per oxygen consumed, as measured by 2′,7′-dichlorofluorescin fluorescence level. This suggests that ALC, by increasing the cellular oxygen consumption, may increase oxidative stress. In the present study, we have demonstrated that, in contrast, 0.15% ALC inhibited oxidative damage to lipids, protein, and nucleic acids. Thus, the oxidative stress increase is a side effect of the high dose of ALC because a low dose of ALC can restore most of the age-associated mitochondrial decay to the young rat level, and endogenous antioxidant defense systems can cope with the increased oxygen consumption. These results also suggest that ALC at low doses, unlike L-carnitine, is effective as an antioxidant.

The more effective action of ALC compared to L-carnitine on cognitive function and oxidative stress may be attributed to the acetyl group or its greater efficiency in crossing the blood-brain barrier.[40] ALC is suggested to donate an acetyl group to choline to form acetylcholine and this hypothesis is the basis of using ALC in the treatment of age-related cholinergic deficits.[61] ALC enhances acetylcholine release in rat striatum and hippocampus[62] and enhances cholinergic synaptic function in old rats.[22] Because transfer of the acetyl moiety from ALC to choline is dependent on the concentration of the ALC and requires the presence of coenzyme A,[63] it may provide an explanation for our observed greater effect of ALC than L-carnitine on cognitive function. The hypothesis that the acetyl group of the ALC molecule is the active group is further supported by the observation that ALC did (but L-carnitine did not) affect the regeneration of the transected sciatic nerve in rats.[64]

In conclusion, ALC and L-carnitine increased ambulatory activity similarly in old rats and elevated carnitine levels in old rat blood and brain. However, L-carnitine did not decrease or enhance oxidative damage, while ALC did decrease MDA, nitrotyrosine, and oxo8dG/oxo8G in old rat brain. These data suggest that ALC is a more effective dietary supplement than L-carnitine.

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