D-Galactose Effectiveness in Modeling Aging and Therapeutic Antioxidant Treatment in Mice

Kodeeswaran Parameshwaran,¹ Michael H. Irwin,¹ Kosta Steliou,² and Carl A. Pinkert¹

Abstract

Accumulating evidence suggests that mitochondrial dysfunction and oxidative stress play major roles in aging. Chronic administration of D-galactose has been reported to cause deterioration of cognitive and motor skills that are similar to symptoms of aging and, therefore, is regarded as a model of accelerated aging. Because enhancing endogenous antioxidants is now widely regarded as an attractive therapy for conditions associated with mitochondrial oxidative stress, in the present study the effects of α -lipoic acid, L-carnitine, and PMX-500F on D-galactose treated mice were tested. Female mice were injected with (100 mg/kg) D-(+)-galactose for 6 weeks and some groups were treated with a daily dose of α -lipoic acid (5 mg/kg), L-carnitine (3.9 mg/kg), PMX-500F (11.9 mg/kg), or the vehicle (0.1 M Tris, pH 7.4). Control mice were treated with physiological saline. An accelerating Rota-Rod, open field test, and Y-maze test were performed, and serum lactate concentrations were analyzed. These analyses did not identify impairment in motor coordination, open-field activity, or spatial memory ($p > 0.05$). Similarly, serum lactate concentrations in D-galactose-treated mice were not elevated when compared to controls ($p > 0.05$). Treatment with the antioxidant compounds at the given concentrations did not result in any changes in the behavioral parameters tested. In conclusion, results of this study illustrate that chronic, short-term D-galactose treatment may not represent a suitable model for inducing readily detectable age-related neurobehavioral symptoms in mice.

Introduction

NORMAL AGING IS ASSOCIATED with gradual, incremental loss of cognitive and motor performances. Yet, in age-dependent neurological disorders, cognitive decline is rapid, resulting in severe disruptions in normal life. Concomitantly, disease pathology often involves common processes, including perturbations in mitochondrial function. Mitochondrial dysfunction characterized by decreased oxidative phosphorylation and increased free radical and reactive oxygen species (ROS) generation was shown to be associated with aging and certain diseases, including Alzheimer's, Huntington's, and Parkinson's diseases.^{1–5} A better understanding of the nature of mitochondrial dysfunction during normal aging and in early phases of neurodegenerative diseases would shed light on the pathogenesis of cognitive decline, paving the way for better therapeutic strategies.

Although many neurological diseases are linked to familial genetic mutations, sporadic and age-related diseases are more common and have lesser understanding of disease mechanisms. Recent reports implicate the chronic administration of D-galactose in accelerating aging, influencing age-

related cognitive decline in mice.⁶ D-Galactose is a physiological nutrient and a reducing sugar that reacts with free amines of amino acids in proteins to form advanced glycation end products through nonenzymatic glycation.⁷ As such, oversupply of D-galactose could contribute to generation of ROS through oxidative metabolism of D-galactose as well as through glycation end products.^{8,9} Interestingly, rodents chronically injected with D-galactose for a period of 6–10 weeks were reported to show progressive decline in learning and memory ability and an increased production of free radicals in brain.⁸

ROS generation in cases of mitochondrial dysfunction is the major cause of intracellular damage. Excessive ROS generation coupled with disrupted antioxidant defenses leads to oxidative damage to mitochondria. Thus, use of antioxidants is regarded as a useful therapy for ROS-induced mitochondrial damage. Indeed, dietary supplements and foods rich in natural antioxidants were shown to be marginally beneficial but with limited bioavailability in animals. One option to increase antioxidant supply and improve bioavailability is to deliver naturally occurring antioxidants to populations of dysfunctional mitochondria.^{10,11} L-Carnitine is a

¹Department of Pathobiology, College of Veterinary Medicine, Auburn University, Alabama.

²PhenoMatriX, Inc., Boston, MA, and Cancer Research Center, Boston University School of Medicine, Boston, Massachusetts.

compound that is biologically active and naturally produced in the liver and kidneys. L-Carnitine is an acyl carrier compound and mediates transportation of medium- and long-chain fatty acids across mitochondrial membranes and facilitates oxidation of transported fatty acids. L-Carnitine also plays a role in transporting intermediate toxic products out of mitochondria.

Another protective molecule is lipoic acid, which shows antioxidant activity in its oxidized and reduced forms.¹² In addition, we have synthesized a novel compound, PMX-500F, which is a lipoylcarnitine derivative.¹³ In the current study, we tested whether chronic infusion of D-galactose would impair motor coordination, locomotor activity, and spatial reference memory. We also tested the effects of L-carnitine, α -lipoic acid, and PMX-500F on control and D-galactose-treated mice.

Materials and Methods

Animals and treatments

Eight-week-old female C57BL/6J mice (JAX, Bar Harbor, ME) were injected intraperitoneally (i.p.) daily with 100 mg/kg D-(+)-galactose (Amresco, Solon, OH) solution dissolved in sterile 0.9% saline or with vehicle (0.9% saline) for 6 weeks. From the start of the fourth week, mice were injected (i.p.) with R-(+)- α -lipoic acid (5 mg/kg), L-carnitine (3.9 mg/kg), PMX-500F (11.9 mg/kg), or with vehicle (0.1 M Tris, pH 7.4) daily until the end of the experimental period. The experimental design consisted of the following eight groups: Saline and Tris (SAL + Tris); saline and lipoic acid (SAL + LA); saline and L-carnitine (SAL + LC); saline and PMX-500F (SAL + PMX); D-galactose and Tris (D-gal + Tris); D-galactose and lipoic acid (D-gal + LA); D-galactose and L-carnitine (D-gal + LC); and D-galactose and PMX-500F (D-gal + PMX). Mice were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited specific pathogen-free barrier facility, in ventilated microisolator-style caging with *ad libitum* access to water and feed on a 14:10 light:dark cycle.^{14,15} All mouse procedures, including euthanasia, conformed to Institutional Animal Care and Use Committee (IACUC) guidelines and the Guide for the Care and Use of Laboratory Animals, under Office for Laboratory Animal Welfare (OLAW) assurance #A3152-01.

Rota-Rod test

Aging is associated with a progressive decline in motor control and coordination in adults.¹⁶ The Rota-Rod test is a widely used experimental method to evaluate locomotor coordination in mice.¹⁷ In this study, locomotor coordination was tested with a five-station accelerating Rota-Rod (model ENV-575M, Med Associates, St. Albans, VT). During the training trial, mice were trained to coordinate themselves on the Rota-Rod at a constant speed of 24 rpm for 2 min. After a 20-min break, mice were trained again with the same speed and time. If any mouse fell during the training sessions, it was immediately placed back on the Rota-Rod. After each trial, the Rota-Rod was wiped with a detergent solution to eliminate any odor from the previous mouse. The testing session consisted of 10 trials for each mouse with 5 trials per day and 30 min interval between trials. Each trial lasted for

10 min; the speed accelerated from 4 to 40 rpm in 5 min. Falling from the apparatus broke an infrared beam and was automatically recorded by Rota-Rod software (Med Associates, SOF-ENV-575).

Open-field test

The open-field test is used to measure exploratory behavior and spontaneous motor activity in rodents.^{18,19} Decreased activity in the central region of the field is an index of increased anxiety.²⁰ Because aging results in reduced activity and increased anxiety, we used the open-field test to analyze if the D-galactose model would exhibit these effects. The open-field apparatus was a four-sided Plexiglas enclosure with an 18- × 16-inch floor dimension and equipped with a photo-beam activity monitoring system (San Diego Instruments, San Diego, CA). Each mouse was placed gently into the apparatus in the center of the field facing away from an observer and allowed to explore the arena for 10 min. Exploration was assessed as number of grids crossed using the photo-beam activity system. Before placing each mouse, the floor and side walls of the apparatus were cleaned with a detergent solution to eliminate any odors from previously tested mice.

Y-maze test

As aging results in memory deficits, impairment of spatial memory was evaluated using a Y-maze test paradigm following chronic D-galactose administration.^{21,22} The Y-maze consisted of three equal-dimension arms with an angle of 120°. The arm closest to the experimenter was defined as the start arm in which mice were placed at the start of each trial. During trial 1 (5 min), the entrance to the novel arm was closed, limiting the mice to exploration of the start arm and the open arm. After a 30-h intertrial interval, mice were allowed to explore all three arms by having the entrance to the novel arm open. Once the mouse had left the start arm, by placing all four paws outside of the arm, exploratory behavior was assessed for 5 min. This assured that preference for the novel arm was not influenced by the time taken to leave the start arm by each mouse. During the test, the time spent in each arm and the numbers of entries into each arm were recorded. An arm entry and exit were defined as when a mouse had placed all four paws into and outside of the arm respectively. Preference for the novel arm was calculated as a discrimination ratio (Novel/[Novel + Other]) for both the time in arms and number of arm entries. Scores greater than 0.5 indicate a preference for the novel arm.²³

Serum lactate measurement

Alzheimer's disease patients were reported to have higher lactate concentrations, and senescence-prone mice were identified with elevated serum lactate levels.^{24,25} Therefore, we measured serum lactate levels to determine if D-galactose treatment would result in elevated lactate levels. Blood was collected from anesthetized mice and was kept at 4°C for 1 h and then centrifuged at 14,000 rpm for 10 min at 4°C in a microfuge. Serum (4 μ L/sample) was subjected to colorimetric analysis using a lactate colorimetric assay kit (ab65331; Abcam, Cambridge, MA) according to the manufacturer's instructions.

Data analysis

Rota-Rod data were subjected to survival analysis and all other data were analyzed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. Statistical significance was accepted at $p = 0.05$.

Results

Rota-Rod test

The effect of D-galactose and treatment of α -lipoic acid, L-carnitine, and PMX-500F on balance and coordination were tested on an accelerating Rota-Rod. The data were analyzed by the survival analysis procedure. When latency to fall for each animal is compared among groups, the D-gal + Tris group was lower than only two groups ($n = 6$; $p < 0.05$): the SAL + PMX group and D-gal + LA group (Fig 1). However, there was no difference between D-gal + Tris and SAL + Tris groups ($n = 6$; $p > 0.05$), suggesting that chronic D-galactose administration does not impair motor coordination in mice. Antioxidants such as lipoic acid and PMX-500F might have a slight advantage in promoting motor function.

Open-field activity

The open-field study results demonstrated that horizontal activities were not significantly different between the control and D-galactose treated mice, and treatment with α -lipoic acid, L-carnitine, or PMX-500F did not influence open-field activity ($F_{7, 32} = 0.54$; $n = 5$; $p > 0.05$; Fig. 2A). In addition, analysis of the activities at the center of the field also shows that there was no difference among the experimental groups, suggestive that D-galactose treatment or α -lipoic acid, L-carnitine, or PMX-500F did not result in a state of anxiety ($F_{7, 32} = 0.44$; $n = 5$; $p > 0.05$; Fig 2B).

FIG. 1. D-Galactose (D-Gal) and motor coordination. D-Galactose administration does not impair motor coordination in mice. Average latency to fall was lower in the D-galactose group when compared to SAL + PMX-500 and D-gal + LA groups (survival analysis; $n = 6$, $p < 0.05$).

Y-maze test

Analysis of data from Y-maze experiments shows that neither the time spent in the novel arm ($F_{7, 32} = 0.40$; $n = 5$; $p > 0.05$; Fig. 3A) nor the number of visits to the novel arm in terms of discrimination ratio were dependent on the experimental treatments ($F_{7, 32} = 1.47$; $n = 5$; $p > 0.05$; Fig. 3B). These results suggest that spatial reference memory and novelty-seeking behavior were not affected in D-galactose mice and the compounds used did not have any discernable effect on these behavioral measures.

Serum lactate

Serum lactate assay results showed that the serum lactate concentrations were similar and did not vary among the experimental groups ($F_{7, 40} = 0.04$; $n = 6$; $p > 0.05$; Fig. 4). These results illustrated that administration of D-galactose did not affect mitochondrial toxicity that would effect an accumulation of lactic acid in the serum.

Discussion

A decline in body functions is observed during aging partly due to impairments in various systems in the brain, including cognitive mechanisms.¹⁶ Brain aging is associated with reduced neurogenesis, increased neurodegeneration, and various other deficits primarily involving the neurotransmitter systems. Accumulating evidence suggests mitochondrial dysfunction potentially contributes to age-associated neuropathologies. Reports have described symptoms of brain aging, including impaired neurogenesis, memory deficits, neurodegeneration, and oxidative damage in rodents as a result of chronic administration of D-galactose.^{26,27} In particular, forms of spatial memory dependent on hippocampal function decreased after chronic D-galactose treatment.²⁸ In this study, motor coordination, locomotor activity, spatial reference memory, and serum lactate concentration were evaluated after chronic D-galactose administration. Concomitantly, the effects of α -lipoic acid, L-carnitine, and PMX-500F with antioxidant properties were characterized in respect to age-related behavioral and biochemical measures.

Studies have shown that motor skills and in particular coordinating skills on the Rota-Rod declined with natural aging.^{29–31} Similar results were reported in rodent models of Alzheimer's disease, Parkinson's disease, and Huntington's disease in which mitochondrial dysfunction and oxidative stress play a central role in disease pathology.^{32,33} However, the results from this study suggest that D-galactose treatment at the chosen dose regimen did not impair motor coordination in mice. To our knowledge, this is the first study that reports motor coordination evaluation in the D-galactose mouse model using the Rota-Rod test. These results suggest that the D-galactose model may not be suitable to study aging-related changes in motor function.

In addition to age-associated motor coordination decline; aging was associated with reduced activity in the open field,³⁴ and in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's disease³⁵ in which mitochondrial apoptotic pathways play an important role in MPTP neurotoxicity.^{36,37} The open-field test was used to assess locomotor activity in an open-field arena, and reduced activity in the center of the field was suggestive of anxiety.²⁰

FIG. 2. D-Galactose (D-Gal), locomotor activity, and open-field testing. D-Galactose administration does not impair the locomotor activity and does not cause increased anxiety in the open-field test. (A) The horizontal activity measured in terms of the numbers of grids crossed was not different among groups (analysis of variance [ANOVA]; $n=5$, $p>0.05$). (B) The number of activities in the center of the open field was not different between groups (ANOVA; $n=5$, $p>0.05$).

However, the open-field data in this report did not identify reduced exploratory locomotor activity associated with D-galactose treatment. In contrast to our results, other reports have documented D-galactose influence and impaired activity in the open field.^{9,28,38,39} Studies that used a high (500 mg/kg per day) D-galactose dose also reported lesser exploratory activities in open-field testing.^{40,41} Interestingly, all of these studies used a Kunming strain of mice. Yet, another more recent study, which used Laca mice, did not find any difference in open-field activity between the D-galactose-treated and control mice.⁴² Taken in the aggregate, open-field behavior might show mouse strain-dependent variation where the D-galactose treatment may not be adequate to mask interstrain variation. This notion is supported by a recent report that identified a robust strain ranking effect on open-field behavior in eight inbred mouse strains.⁴³ This is the first study to report on anxiety evaluation in the D-galactose model. Previous studies used open-field test for exploratory behavior, and did not evaluate anxiety by analyzing the time spent in the center region of the field.

Aging and many age-related neurological diseases are characterized by memory deficits. Our results with Y-maze testing demonstrate that the discrimination ratio for time spent in the novel arm, as well as the number of entries into the novel arm, were not reduced in D-galactose-treated mice. In addition, all groups showed a discrimination ratio greater than 0.5; suggestive that mice showed preference for the novel arm with unimpaired novelty-seeking behavior and spatial memory.²³ The Y-maze experimental paradigm was designed to assess the short-term spatial reference memory that relies on the novelty-seeking behavior of the mice tested. Still, other studies reported impaired spatial memory in D-galactose-treated mice when assessed with the Morris water and Y-mazes.^{42,44–48} Though most of the studies on D-galactose induced learning and memory impairment were performed with Kunming mice, interestingly, only one study reported deficits in learning and memory in C57BL/6 mice using a Morris water maze analysis.⁴⁹ Yet, in agreement with our results, novelty-seeking behavior was not affected.⁴⁴ These results lend further support to our

FIG. 3. D-Galactose (D-Gal) and spatial memory. Chronic treatment of D-galactose did not result in impaired spatial memory. (A) Discrimination ratio for the time spent in the novel arm was not significant among groups (ANOVA; $n=5$, $p>0.05$). (B) Discrimination ratio for the number of entries into the novel arm was not different between groups (ANOVA; $n=5$, $p>0.05$).

FIG. 4. Serum lactate in D-galactose-treated mice. Serum lactate concentrations were not altered in mice treated with D-galactose. Serum lactate concentrations were measured with a colorimetric assay and the results illustrate no differences between groups (ANOVA; $n = 6$, $p > 0.05$).

contention that D-galactose administration may not be suitable to model experimental aging-induced memory deficits, particularly in experimental paradigms that do not involve reward/penalty stimuli (as in the case of forced swimming in Morris water maze).

Last, serum lactate measurements were used to indicate whether chronic D-galactose treatment resulted in lactic acidosis by systemically altering oxidative metabolism of mitochondria. Surprisingly, D-galactose treatment did not increase serum lactate concentration, and there were no differences between antioxidant treatment groups. This result indicated that anaerobic metabolism was not elevated, which might occur if mitochondrial respiration was compromised. Increased serum lactate content was reported in a senescence-prone mouse model and was believed to reflect an impaired brain glucose metabolism.²⁵ Higher levels of serum lactate content were also reported in severely cognitive-deficient dogs and in early-onset Alzheimer disease patients.²⁴ Taken together, one may surmise that D-galactose administration may not result in significant lactic acidosis.

In conclusion, the present study evaluated the D-galactose model of accelerated aging; the effects of selected antioxidants on age-related neurobehavioral outcomes and mitochondrial oxidative metabolism were also analyzed. Results in this report illustrate that D-galactose administration does not elicit lactic acidosis, does not result in altered motor or memory function, and, therefore, appears unsuitable for testing antioxidant compounds in the treatment of mitochondrial perturbations related behavioral, neurological aging, and mitochondrial diseases. Accordingly, it is our hypothesis that mice with specific mutations in nuclear or mitochondrial genomes may provide more reliable models to study the neurological effects of perturbations in mitochondrial function and protective efficacies of antioxidants.

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Author Disclosure Statement

The authors have no competing financial interests or commercial associations; no competing financial interests exist.

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Address correspondence to:

Dr. Carl A. Pinkert
202 Samford Hall
Auburn University
Auburn, AL 36849-5112

E-mail: cap@auburn.edu

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