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Antioxidant treatment with phenyl-α-*tert*-butyl nitrone (PBN) improves the cognitive performance and survival of aging rats

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Abstract

Accumulating evidence has implicated free radical production and resultant oxidative damage as a major contributing factor in brain aging and cognitive decline. In the present study, aging 24-month-old rats were chronically treated with the synthetic spin-trapping antioxidant phenyl-α-*tert*-butyl nitrone (PBN) for up to 9.5 months. Chronic PBN treatment (1) improved the cognitive performance of aged rats in several tasks, (2) resulted in greater survival during the treatment period, and (3) decreased oxidative damage within brain areas important for cognitive function. These results not only provide a direct linkage between free radicals/oxidative damage and cognitive performance in old age, but also suggest that synthetic brain antioxidants could be developed to treat or prevent age-associated cognitive impairment and Alzheimer's disease.

Keywords: Antioxidants; Phenyl-α-*tert*-butyl nitrone (PBN); Aging rats; Cognition; Survival; Free Radicals; Oxidative damage; Alzheimer's disease

Numerous theories currently exist which attempt to explain both the cognitive decline and physical changes associated with aging. Of increasing interest is the 'Free Radical Hypothesis of Aging', which maintains that (1) endogenous antioxidant defense mechanisms are insufficient to detoxify all oxygen free radicals continually being generated, and (2) resulting oxidative damage to critical biological molecules such as DNA, protein, and membrane lipids contributes to age-related neuronal loss and/or dysfunction [6]. Supportive of this hypothesis are recent studies showing increased oxidative damage during normal brain aging, as well as in Alzheimer's disease and Parkinson's disease [10,12]. If, in fact, oxygen free radicals (such as superoxide and hydroxyl radicals) are important causative factors in normal brain aging and neurodegenerative diseases, chronic enhancement of antioxidant defenses should slow this process, resulting in improved cognitive and/or motor function.

One synthetic antioxidant capable of scavenging many types of free radicals, including both oxygen- and carbon-based radicals, is the spin trapping compound phenyl-α-*tert*-butyl nitrone (PBN). Because PBN is both hydrophilic and lipophilic, it readily permeates all tissues, including the brain; its half-life in blood is 3–4 h [3,4]. By adding free radicals to its carbon-nitrogen double bond, PBN detoxifies them, yielding a stable nitroxide product which is safely metabolized and excreted in the urine [3, 4,8]. In a recent study [11], we found that chronic treatment with PBN, in combination with the antioxidants α-tocopherol (vitamin E) and ascorbate (vitamin C), enhanced both acquisition and memory retention of aged rats in water maze performance. The present study extends these initial findings by showing that long-term treatment of aged rats with PBN alone results in (1) improved cognitive performance in two diverse tasks, (2) increased survival, and (3) reduced oxidative damage within cognitively-important brain areas.

Inbred male Sprague–Dawley rats of our own aging colony were housed individually, with free access to food/water, and kept on a 14/10 h light-dark cycle. One group of 24-month-old rats ($n = 11$; 591 ± 14 g) was begun on daily i.p. treatment with 32 mg/kg PBN (made fresh daily), while another group of 24-month-old control animals ($n = 12$; 594 ± 18 g) was begun on daily i.p. treatment with 0.9% saline vehicle solution. Two months into treatment, surviving animals were tested for acquisi-

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tion/memory retention in the Morris water maze (see below). At 4 months into treatment, animals were further tested for acquisition (learning) of one-way active avoidance pole jumping (see below). PBN or vehicle treatment was continued through 33.5 months of age and survival of animals in both groups was monitored through the 9.5-month treatment period. Animals surviving through 33.5 months of age were sacrificed by decapitation, as were animals at least 30 months of age that were clearly near death. No significant differences in weight were present between PBN- and vehicle-treatment groups at weekly weighings during the study and group weights at sacrifice were also not significantly different from one another (518 ± 27 versus 522 ± 25 g). The following brain areas were discretely dissected out for determination of basal levels of lipid peroxidation [11], as determined by thiobarbituric acid reactive product (TBAR) formation: frontal cortex, parietal cortex, neostriatum, globus pallidus, dorsal hippocampus, and substantia nigra. Non-specific incubation-induced TBAR formation was eliminated by inclusion of the iron chelator desferrioxamine and butylated hydrotoluene (BHT) in the tissue homogenation's chemical mixture.

Water maze testing was done as previously described [11], with the submerged transparent 'escape' platform placed in the center of Quadrant 2 (Q2). 'Acquisition' (learning) was tested over 18 days and consisted of four consecutive daily trials, with the animal placed once in each of the four quadrants to initiate a trial. Latency to reach the submerged platform in Q2 was determined and an average latency of each day's trials was calculated. Two-way analysis of variance for repeated measures (ANOVARM) was used to compare acquisition data from antioxidant and control groups. On the day following completion of acquisition testing, animals were given a single 60 s 'memory retention' (probe) trial, in which the platform was removed and animals were placed in the quadrant (Q1) opposite the platform quadrant. Percentage of time spent in the quadrant formerly containing the platform (Q2) was used as a measure of memory retention. A separate group of six 27–28 month-old 'naive' rats (i.e., no acquisition training) was tested in the retention trial to determine any quadrant bias inherent to the retention testing procedure.

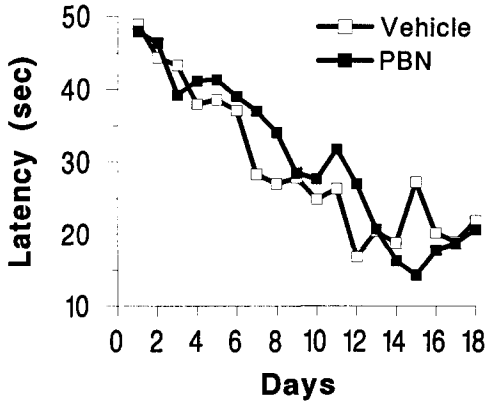

Fig. 1. Acquisition (learning) in the Morris water maze for PBN-treated ($n = 10$) and vehicle-treated ($n = 9$) aged rats 2 months into treatment. Latency to find the submerged platform in Quadrant 2 is shown over 18 days of testing.

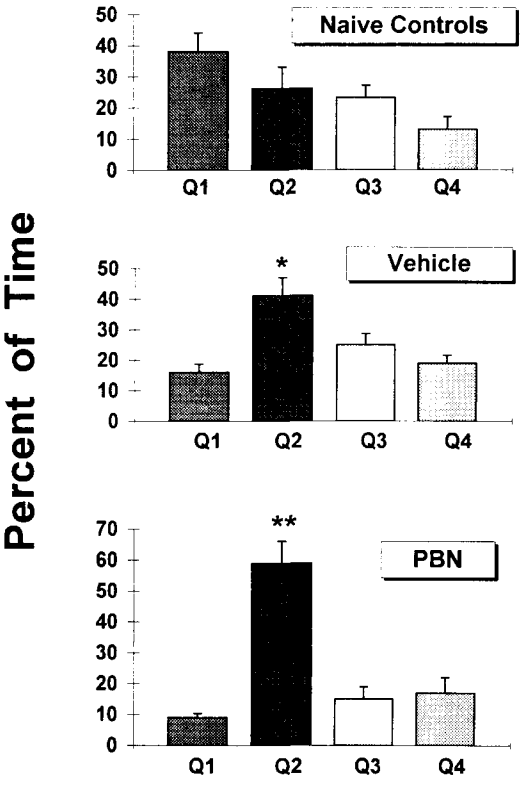

Fig. 2. Memory retention in the Morris water maze for naive controls ($n = 6$), vehicle-treated ($n = 9$), and PBN-treated ($n = 10$) aged rats showing percentage of time spent in each of the circular pool's equally-sized four quadrants (Q1, Q2, Q3, and Q4) during a 1-min free swim. Quadrant 2 (Q2) formerly contained the platform. Bars represent mean ± SEM. *Significantly different from the other three quadrants of that group. **Significantly different from the other three quadrants of that group and also significantly different from Q2 for the other groups. Data from the naive control group was previously published in [11].

For pole jumping active avoidance testing, an overhead lamp served as the conditioning stimulus. The light was turned on for 5 s, followed by a 15 s foot shock of 2–4 mA delivered through a metal grid floor. If the animal jumped onto the pole within the 5-s delay, he avoided the shock (a conditioned avoidance response or CAR). Subjects were given ten trials per day for 3 days of shaping, followed by 9 days of testing. For each animal, a percentage of CARs was determined daily from the ten trials given. Two-way ANOVARM was used for group comparisons.

PBN and vehicle treatment groups both showed an improvement in water maze acquisition (learning) over time ($P < 0.001$), although overall acquisition and rate of acquisition were not significantly different between these

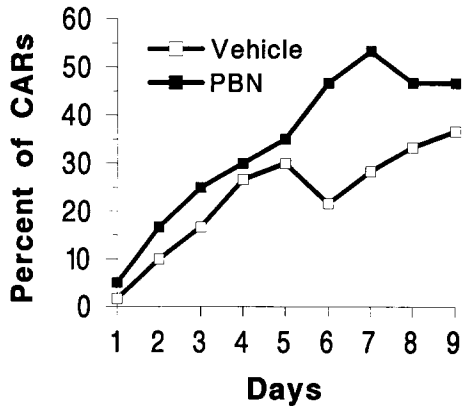

Fig. 3. One-way active avoidance acquisition (learning) for PBN-treated ($n = 6$) and vehicle-treated ($n = 6$) aged rats at 4 months into treatment. The percent of CARs from ten trials is shown over 9 days of testing.

groups (Fig. 1). For the memory retention phase of testing (Fig. 2), ANOVA tests indicated that both PBN- and vehicle-treated animals had a quadrant preference; post-hoc Student t-tests revealed that both groups spent a higher percentage of swim time in Quadrant 2 compared to the other three quadrants ($P < 0.001$ and $P < 0.05$, respectively). However, PBN-treated animals spent significantly more time in the quadrant formerly containing the platform in comparison to either vehicle-treated or naive rats ($P < 0.05$; Student's t-test), indicating significantly better memory retention for PBN-treated animals. In the test for acquisition of one-way active avoidance behavior 4 months into treatment, PBN-treated animals had significantly greater overall acquisition ($P < 0.02$) compared to vehicle-treated animals (Fig. 3); there was no difference in the rate of acquisition between these groups.

Animals treated with PBN had significantly higher survival (lower mortality) than vehicle-treated controls during the 9.5-month study period from 24 to 33.5 months of age (Fig. 4), as indicated by the log-rank test for survivorship ([1]; $\chi^2(1) = 4.74$; $P < 0.05$). At 32 months into the study, seven of 11 PBN-treated animals were still alive, compared to only one of 12 vehicle-treated animals, a significant difference as revealed by χ^2 analysis ($\chi^2(1) = 7.86$; $P < 0.01$). Furthermore, average life-span of PBN-treated animals was significantly increased by at least 10% compared to controls (30.9 ± 0.9 versus 28.0 ± 0.8 months; $P < 0.03$). Because 36% of PBN animals (but only 8% of controls) were alive at the end of the study period, an extension of the study period would have almost certainly resulted in an even greater effect of PBN treatment on average life-span and a possible increase in maximum life-span as well. Of the six brain regions analyzed for 'basal' TBAR formation, PBN treatment for over 9 months significantly reduced TBAR formation in frontal cortex ($P < 0.05$), parietal cortex ($P < 0.01$) and globus pallidus ($P < 0.001$) compared to controls (Fig. 5).

Although numerous studies have indicated an association between free radicals/oxidative damage and brain aging [12], the enhanced cognitive performance of aged rats provided by chronic administration of the antioxidant PBN in the present study provides a direct linkage between free radicals/oxidative damage and cognitive performance in old age. The improved cognitive performance shown by antioxidant-treated animals in the present study is consistent with an earlier study showing that rats chronically maintained on a low antioxidant diet (i.e., no α-tocopherol) had impaired cognition [9].

PBN treatment that began at 24 months of age enhanced the survival of aging rats over a 9.5-month study period. Previously, life-long 'dietary' administration of antioxidants such as 2-mercaptoethylamine, ethoxyquin,

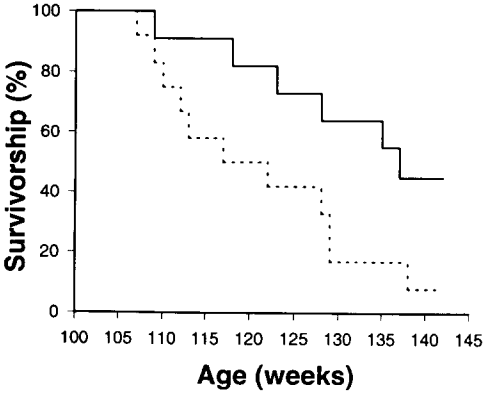

Fig. 4. The percent of surviving PBN-treated rats (solid line) and vehicle-treated rats (dashed line) at weekly intervals over a 9.5-month treatment period from 24–33.5 months of age. At the beginning of treatment, $n = 11$ and $n = 12$ for PBN- and vehicle-treatment groups, respectively. Log-rank analysis of survivorship indicated a significantly increased survival of PBN-treated animals compared to controls.

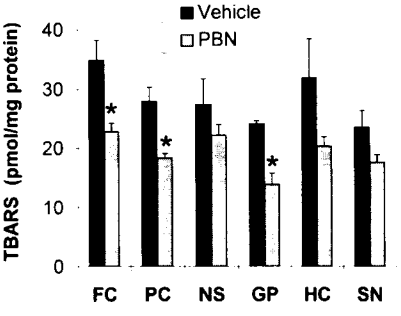

Fig. 5. Basal levels of lipid peroxidation, as indexed by TBAR formation, in six brain regions from aged control rats and those given at least 9 months of PBN treatment. Bars represent mean ± SEM for 4–7 animals. *Significantly different from control group at $P < 0.05$ or greater level of significance. Abbreviations: FC, frontal cortex; GP, globus pallidus; HC, dorsal hippocampus; NS, neostriatum; PC, parietal cortex; SN, substantia nigra.

and butylated hydroxytoluene (BHT) has been shown to extend the life-span of mice [2,5,7]. In such studies, dietary antioxidant treatment began at an early age (i.e., 1–4 months old). However, the present study in rats demonstrates for the first time that antioxidant treatment beginning later in life (at the human equivalent of 50–60 years of age) can increase life-span. The mechanism(s) by which such PBN treatment increases survival and cognitive function during aging most likely involves an enhancement of endogenous antioxidant defenses to limit ongoing oxidative damage. In this context, the 'spin-trapping' nature of PBN quenches a variety of oxygen- and carbon-based free radicals [3,4,8]. Supportive of this premise, PBN treatment for over 9 months in the present study resulted in reduced lipid peroxidation within two brain areas important for cognitive function, the neocortex and globus pallidus (containing the nucleus basalis).

In the present study, the ability of chronic antioxidant treatment with PBN to enhance cognitive performance and survival in aging rats suggests that there is an effect on the underlying process of aging, thus providing support for the Free Radical Hypothesis of Aging [6]. In view of the increasing evidence for involvement of free radicals/oxidative damage in brain aging and Alzheimer's disease [10,12], the development of synthetic antioxidants could represent a viable therapeutic approach to treat or prevent the cognitive impairment associated with aging and Alzheimer's disease.

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