JOURNAL OF ANTI-AGING MEDICINE
Volume 2, Number 4, 1999
Mary Ann Liebert, Inc.

Effect of Carnosine on Age-Induced Changes in Senescence-Accelerated Mice

M.O. YUNEVA,[1] E.R. BULYGINA,[1] S.C. GALLANT,[2] G.G. KRAMARENKO,[3] S.L. STVOLINSKY,[3] M.L. SEMYONOVA,[4] and A.A. BOLDYREV[1,3]

ABSTRACT

The effect of carnosine on the life span and several brain biochemical characteristics in senescence-accelerated mice–prone 1 (SAMP1) was investigated. A 50% survival rate of animals treated with carnosine increased by 20% as compared to controls. Moreover, the number of animals that lived to an old age significantly increased. The effect of carnosine on life span was accompanied by a decrease in the level of 2′-tiobarbituric acid reactive substances (TBARS), monoamine oxidase b (MAO b), and Na/K-ATPase activity. There was also an increase in glutamate binding to *N*-methyl-D-aspartate receptors. These observations are consistent with the conclusion that carnosine increases life span and quality of life by diminishing production of lipid peroxides and reducing the influence of reactive oxygen species (ROS) on membrane proteins.

INTRODUCTION

SENESCENCE-ACCELERATED MICE–PRONE 1 (SAMP1) show rapid aging, which has been attributed to genetic deficits of metabolism, resulting in overproduction of reactive oxygen species (ROS).[1–4] We have designed experiments to test the effects of carnosine, a natural hydrophilic antioxidant, on life span and several biochemical indicators known to change with age, studied in the SAMP1 strain.[1]

Carnosine has been demonstrated to be a protector of neurons from oxidative stress, caused by excitotoxic compounds *in vitro*[5] or from experimental hypoxia *in vivo*.[6,7] The effect of carnosine may be specific, in that neither of its component amino acids, histidine and β-alanine, show similar actions.[8]

MATERIALS AND METHODS

Animals

SAMP1 animals were kept under standard laboratory conditions and fed a balanced diet. A distinct excess in signs of aging by the age of 8–10 months was observed for SAMP1 mice in comparison with SAMR1 (senescence-accelerated mice–resistant 1); this was reflected in both the physical features and the behavioral reaction of SAMP1 strain.[1] The maximal life

[1]International Centre for Biotechnology and Centre for Molecular Medicine, Department of Biochemistry, School of Biology, MV Lomonosov State University, Moscow, Russia.
[2]Extend Foundation, Herts, United Kingdom.
[3]Institute of Neurology, Russian Academy of Medical Science, Moscow, Russia.
[4]Department of Embryology, School of Biology, MV Lomonosov State University, Moscow, Russia.

span of SAMP1 mice was 15 months in our conditions, whereas that for SAMR1 mice was 24 months.[1] SAMP1 mice of both sexes were randomly divided into two groups of 70 animals each at the age of 2 months. For one group, carnosine was added to the drinking water in the amount corresponding to 100 mg/kg body weight per day. A 50% survival rate was determined by following survival until the age of 15 months, estimating mortality and accumulation of age changes using the Grading Score System (GSS).[9]

Methods

At an age of 10 months, distinct features of aging appeared in the SAMP1 animals. At this time, some animals were decapitated, and total microsomal, mitochondrial, and synaptosomal fractions were prepared from cortex.

Preparation of membrane fractions. Tissue samples were homogenized in 10 mM potassium phosphate buffer (pH 7.4). Homogenates were centrifuged at 10,000*g* for 30 min. The total microsomal fraction was prepared as described elsewhere[6] and used for measurement of malonic dialdehyde (MDA) as an indicator of the oxidizing capacity of brain membranes. Synaptosomes were prepared from brain cortex according to Hajos.[10] Samples were homogenized in a medium containing 10 mM HEPES, 0.32 M sucrose, and 2 mM EDTA (pH 7.4). Initial microsomes were prepared by differential centrifugation. The synaptosomal fraction was obtained from the last sucrose density gradient centrifugation (1 part of 0.32 sucrose to 4 parts of 0.8 mM sucrose). It was washed in distilled water and suspended in a medium containing 30 mM histidine, 1 mM EDTA, and 0.25 mM sucrose (pH 7.4). Samples were stored at −70°C before analysis.

Biochemical parameters. Na/K-ATPase activity in the synaptosomal fraction was measured by inorganic phosphate liberation.[11] The reaction was carried out in the medium containing 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3 mM ATP, and 30 mM PIPES (pH 7.4 at 37°C). Na/K-ATPase activity was calculated as the difference between activities measured in the presence of 130 mM NaCl and 20 mM KCl and those measured in NaCl-free medium, containing 150 mM KCl.

Before radioligand binding analyses, the protein samples (60–120 μg) were solubilized with 0.05% Tritone X-100 with subsequent twofold washing. Total glutamate binding was determined in the presence of unlabeled glutamate (200 nM), in the medium containing 10 nM [^{3}H]Glu, 1 μM glycine, and 50 mM Tris-acetate buffer (pH 7.4 at 4°C). Nonspecific binding was defined by 1 mM *N*-methyl-D-aspartate (NMDA), and maximal value for NMDA-receptor binding (B_{max}) was estimated as the difference between these two values.[12]

Mitochondria were prepared from the brain homogenate, suspended in 0.2 M K/Na-phosphate buffer (pH 7.4 at 4°C), and stored at −70°C before measurement of monoamine oxidase b (MAO b) activity.[1] MAO b activity in mitochondrial fraction was measured by accumulation of benzaldehyde. The reaction was initiated by the addition of benzylamine (1 mM) and stopped by the addition of 5% trichloroacetic acid (final concentration) after 2 h of incubation at 37°C. To control samples, benzil amine was added after the reaction was stopped. Accumulation of benzaldehyde was measured against the control sample after their extraction in hexane. The measurements were made at 241 nm using LKB spectrophotometer Pharmacia LKB-Ultrospec III (molar extinction coefficient of benzaldehyde in hexane, ε = 13,080 $M^{-1}cm^{-1}$).

Lipid peroxidation (LPO) in brain microsomes was determined by MDA accumulation, measured as a 2′-thiobarbituric acid reactive substance (TBARS) Molar extinction coefficient at λ = 535 nm is 1.56×10^5 M^{-1}.[13] The initial level of MDA as well as its accumulation after 90 min of LPO induced by $FeSO_4$ (10 mM) and ascorbic acid (200 μM) were measured.[6]

Statistical analysis. All parameters measured were expressed as mean ± SEM calculated from no less than three samples. Statistical analysis was carried out using a routine computer program, and p values of <0.05 were considered to be statistically significant. GSS parameters were analyzed using the Students' paired t test.[1,9]

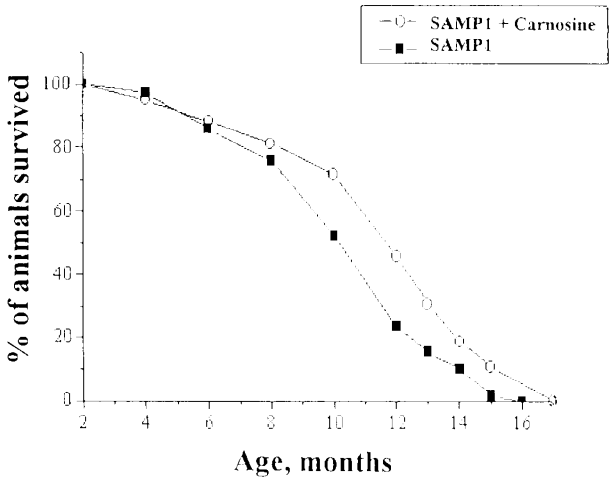

FIG. 1. Life span of SAMP1 animals treated and nontreated with carnosine. Each group analyzed contained 70 animals.

RESULTS

When SAMP1 animals were given carnosine in their diet, their maximal life span did not significantly increase, but 50% survival rate increased by 20%, because there was an increase in the number of animals living to older ages (Fig. 1). Carnosine had a clear effect on external appearance of animals in that the coat fullness and color were much closer to that of normal animals (Fig. 2). Table 1 shows the parameters measured. Some but not all indicators of aging were significantly retarded by carnosine, including hair glossiness, appearance of skin ulcers, periophthalmic lesions, spinal lordokyphosis, and behavioral responses such as activity and passive avoidance. There was no effect on other parameters, such as body weight, loss of hair, coarseness of hair, and presence of corneal opacities. On the basis of the GSS parameters, these animals met the criteria for being more resistant to features of aging.

Table 2 shows results of the biochemical measurements in carnosine-treated animals and controls. Brain membranes of carnosine-treated animals had lower MDA levels, both when measured initially (decreased by 35%) and after 90 min of induced lipid peroxidation

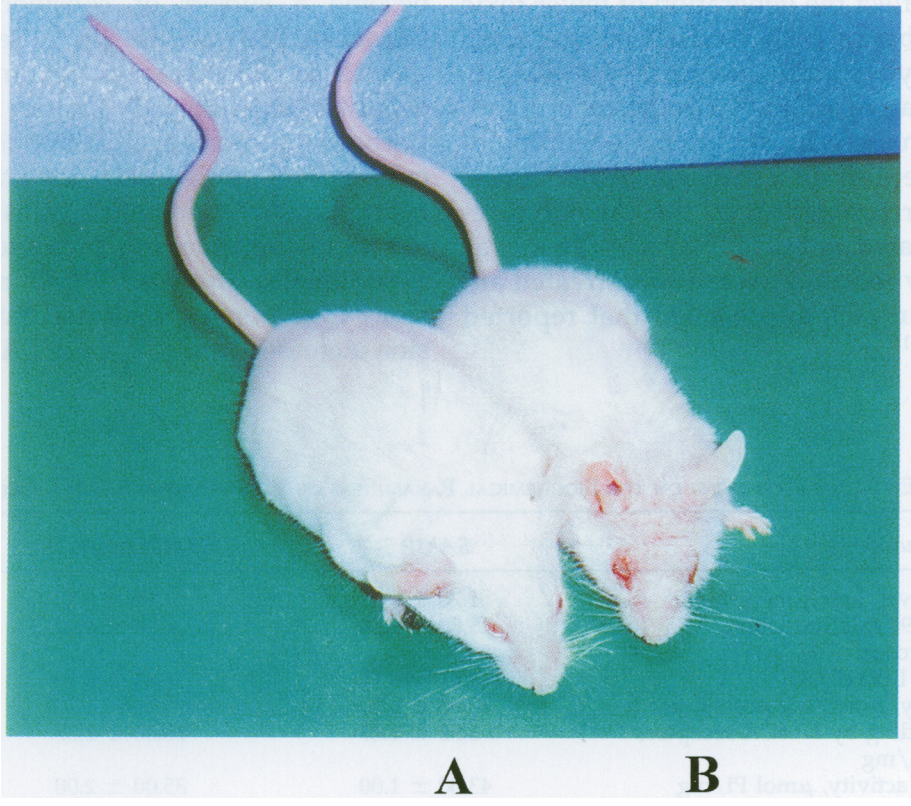

FIG. 2. Exterior of 9-month-old SAMP1 animals treated (**A**) and nontreated (**B**) with carnosine.

TABLE 1. EFFECT OF CARNOSINE ON THE MORPHOLOGICAL AND PHYSIOLOGICAL PARAMETERS OF 8–10-MONTH-OLD SAMP1 ANIMALS[a]

Parameter measured	*SAMP1 (n = 36)*	*SAMP1+carnosine (n = 36)*
Skin and hair		
Loss of hair	91 ± 1	92 ± 2
Loss of glossiness	95 ± 1	56 ± 7***
Coarseness	73 ± 2	78 ± 2
Skin ulcers	36 ± 3	14 ± 2***
Eyes		
Corneal opacity	48 ± 2	45 ± 2
Periophthalmic lesions	92 ± 1	78 ± 2***
Spine lordokyphosis	83 ± 3	72 ± 3*
Physiological behavior		
Reactivity	9 ± 2	58 ± 4***
Passive avoidance	17 ± 2	23 ± 1**
Body weight	29.0 ± 0.6	28.0 ± 0.7

[a]Criteria used are from the test system for grading score of senescence (percentage of animals in a group corresponding to every criterion).
*$p < 0.02$; **$p < 0.01$; ***$p < 0.001$.

(decreased by 30%). However, the relative induction of MDA after $FeSO_4$ (value at 90 min over that initially) was not significantly changed.

Mitochondrial MAO b activity was decreased by 44% in carnosine-treated mice. This may reflect the generation of fewer toxic radicals in brain: both products of the reaction—aldehydes and peroxides—are the factors of oxidative stress.[1,8] On the other hand, there was an increase in glutamate binding to NMDA receptors in carnosine-treated mice, possibly corresponding to the greater reactivity in treated animals. Na/K-ATPase was significantly reduced in carnosine-treated animals and, in fact, approached that reported for SAMR1.[1]

DISCUSSION

Because carnosine is an endogenous compound present in high concentration in a number of tissues, including brain, its action on biological systems and pathologic insults has become a subject of considerable interest. Carnosine has been shown to delay cell aging as well as to increase maximum cell division capacity in cultured human fibroblasts.[14] Carnosine protects against protein glycation in cultured rat fibroblasts.[15] In both brain and heart, carnosine protects against the harmful effects of ischemia and promotes viability in experimental animals.[6–8,16] Our experiments show that long-term administration of carnosine increases the life span of SAMP1 animals.

TABLE 2. EFFECT OF CARNOSINE ON THE BIOCHEMICAL PARAMETERS OF 10-MONTH-OLD SAMP1 ANIMALS (*N = 5*)

Parameter measured	*SAMP 1*	*SAMP1+carnosine*	p
Initial MDA level, nmol/mg protein	1.70 ± 0.18	1.10 ± 0.20	<0.05
MDA level to 90 min induction, nmol/mg protein	5.80 ± 0.60	4.00 ± 0.20	<0.01
Inducibility of LPO (B/A)	3.41 ± 0.11	3.64 ± 0.12	>0.01
MAO b activity, μmol/mg protein per h	57.60 ± 6.50	32.40 ± 1.20	<0.01
Glutamate binding by NMDA-receptors (B_{max}), pmol/mg	6.90 ± 1.03	15.80 ± 2.37	<0.01
Na/K-ATPase activity, μmol Pi/mg protein per h	43.00 ± 1.00	35.00 ± 2.00	<0.05

Carnosine was originally described in brain. In rodents, >70% of ingested carnosine is absorbed and deposited in tissues, particularly muscle and brain.[8] Although carnosine is readily metabolized in the body, its effects after injection are prolonged.[17] This long-lasting action cannot be explained by the appearance of degradation products, since neither histidine nor β-alanine show similar effects, and high doses of histidine actually depress physiological activity.[8,18,19]

The mechanism of the protective effect of carnosine in SAMP1 animals is still uncertain. Carnosine clearly has antiradical activity. It both inhibits lipid peroxidation of brain microsomes and decreases mortality of animals subjected to experimental brain ischemia.[5] These and other observations[20–22] are consistent with a role for endogenous carnosine as a free radical scavenger.

Our observations with MDA and MAO b suggest the possibility that carnosine not only scavenges ROS but also may reduce ROS production. Carnosine treatment suppressed MDA accumulation in brain membranes, but also suppressed its production in response to *in vitro* stimulation of LPO (Table 2). The activity of MAO b, a major generator of ROS, is significantly higher in SAMP1 brains than in SAMR1,[1] but, after treatment with carnosine, falls to levels approaching those in the SAMR1 controls.[1]

The effects of carnosine on glutamate binding and Na/K-ATPase activity are not easily explained by any of the above mechanisms. It is possible that carnosine has effects on expression of different proteins through mechanisms presently not understood, but independent of its antioxidant activity. In addition, carnosine has been reported to have a rejuvenating effect in cell cultures, indicating an ability to affect protein synthesis.[14,15,23]

Aging is associated with accumulation of nonenzymatic glycation of proteins, followed by formation of Amadori products and cross-linkages between macromolecules. These modified molecules lose ability to function normally, but may be more stable against proteolysis. Carnosine has been shown to reduce or prevent protein glycation in a number of model systems,[15,24,25] perhaps because carnosine can itself react with the carbonyl groups of damaged proteins, resulting in complex formation and subsequent removal of glycosyl radicals from the protein molecule.[25] This may explain the restorative effect of carnosine on crystalline lens.[20] The cross-linking of lens proteins results in development of cataracts, but carnosine acts to reverse this process more rapidly than can be explained by new protein synthesis. Thus, carnosine possesses not only an antioxidant effect but may also demonstrate other abilities, including prevention of glycation processes, which may protect proteins against aldehyde attack involving the senescence reactions.

Although from the present data we cannot determine which of the mechanisms of protective effect of carnosine—ROS scavenging, reduced ROS production, antiglycation activity, or some other effect—plays the major role in the model of accelerated senescence we studied, it is noteworthy that the result of carnosine treatment is significant increase of the 50% survival rate of SAMP1 animals.

ACKNOWLEDGMENTS

We are greatly indebted to Professor T. Takeda and Professor M. Hosokawa (Council for SAM Research, University of Kyoto, Japan) for their friendly gift of SAM animals. The work is supported by the Extend Foundation (Hertz, U.K.) and the Russian Foundation for Basic Researches (96-04-49078).

REFERENCES

1. Bulygina E, Gallant S, Kramarenko G, Stvolinsky S, Yuneva M, Boldyrev A. Characterization of the age changes in brain and liver enzymes of senescence accelerated mice (SAM). *J Anti-Aging Med* 1999;2:45–49.
2. Yokoi I, Inada K, Habu H, et al. Age-related changes in nitric oxide content and nitric oxide synthase activity in senescence-accelerated mouse (SAMP8) brain. In: Cutler RG, Packer L, Bertram J, Mori A, eds. *Oxidative Stress and Aging*. Basel: Berkhauser Verlag, 1995:387–393.
3. Hosokawa M, Abe T, Higuchi K, et al. Management and design of the maintenance of SAM mouse strains: an animal model for accelerated senescence and age-associated disorders. *Exp Gerontol* 1997;32:111–117.

4. Warner HR, Starke-Reed P. Oxidative stress and aging. In: Clerch LB, Massaro DJ, eds. *Oxygen, Gene Expression, and Cellular Function.* New York: Marcel Decker, 1997:139–167.
5. Boldyrev AA, Lawrence D, Carpenter D. Effect of carnosine and its natural derivatives on apoptosis of neurons induced by excitotoxic compounds. In: Shimanishi Y, ed. *Peptide Science—Present and Future.* Boston, Dordrecht, London: Kluwer Academic Publishers, 1998:424–426.
6. Boldyrev AA, Kukley ML, Stvolinsky SL, Gannushkina IV. Carnosine and free radical defense mechanisms in the brain. In: Packer L, Traber MG, Xin W, eds. *Proceedings of the International Symposium on Natural Antioxidants: Molecular Mechanisms and Health Effects.* Champaign, IL: American Oil Chemists Society, 1996:600–614.
7. Boldyrev AA, Stvolinsky SL, Tyulina OD, Koshelev VV, Hori N, Carpenter DO. Biochemical and physiological evidence that carnosine is an endogenous neuroprotector against free radicals. *Cell Mol Neurobiol* 1997;17:259–271.
8. Boldyrev AA. *Carnosine* [in Russian] Moscow: Moscow University Publishing House, 1998.
9. Takeda T, Hosokawa M, Takeshita S, et al. A new murine model of accelerated senescence. *Mech Aging Dev* 1981;17:183–194.
10. Hajos F. An improved method for the preparation of synaptosomal fraction of high purity. *Brain Res* 1975;93:334–336.
11. Klodos I, Ottolenghi P, Boldyrev A. Large-scale preparation of Na/K-ATPase from ox brain. *Anal Biochem* 1975;67:397–403.
12. Kyle PJ, Raymond JP, Karbon W, Ferkany JW. NMDA receptors: heterogeneity and agonism. In: Krogsgaard-Larsen P, Hansen JJ, eds. *Excitatory Amino Acid Receptors.* New York: Ellis Horwood, 1999:121–161.
13. Slater TF. Overview of methods used in detecting lipid peroxidation. *Methods Enzymol* 1984;105:283–293.
14. McFarland GA, Holliday R. Retardation of the senescence of cultured human diploid fibroblasts by carnosine. *Exp Cell Res* 1994;212:167–175.
15. Kanta SS, Wada S, Tanaka H, et al. Carnosine sustains the retention of cell morphology in continuous fibroblast culture subjected to nutritional insult. *Biochem Biophys Res Commun* 1996;223:278–282.
16. Alabovsky VV, Boldyrev AA, Vinokurov AA, Shchavratsky VKH. Effect of histidine-containing dipeptides on isolated heart under ischemia/reperfusion. *Biochemistry (Moscow)* 1996;62:91–102.
17. Stvolinsky SL, Sousa-Ponyesh E, Sergienko VI, Boldyrev AA. Immunomodulating effect of carnosine in the *in vitro* and *in vivo* experiments [in Russian]. *Biol Membranes (Moscow)* 1996;13:299–306.
18. Orthen-Cambill N. Antihistaminic drugs increase feeding, while histidine suppresses feeding in rats. *Pharmacol Biochem Behav* 1988;31:81–86.
19. Dutra-Filho CS, Wannmacher CM, Pires RF, et al. Reduced locomotor activity of rats made histidinemic by injection of histidine. *J Nutr* 1989;119:1223–1227.
20. Boldyrev AA, Dupin AM, Bunin AY, Babizhaev MA, Severin SE. The antioxidant properties of carnosine, a natural histidine-containing dipeptide. *Biochem Int* 1987;15:1105–1113.
21. Kohen R, Yamamoto Y, Kundy KS, et al. Antioxidant activity of carnosine, homocarnosine, and anserine present in muscles and brain. *Proc Natl Acad Sci USA* 1988;85:3175–3179.
22. Aruoma OL, Laughton MJ, Halliwell B. Carnosine, homocarnosine and anserine: could they act as antioxidants *in vivo? Biochem J* 1989;264:863–869.
23. Hipkiss AR, Michaelis J, Syrris E, et al. Non-enzymatic glycosylation of the dipeptide carnosine, a potential anti–protein cross-linking agent. *FEBS Lett* 1995;371:81–85.
24. Boldyrev A, Carpenter D, Kovalenko Z, Kuleva N, Lawrence D, Song R. Carnosine protects neurons from excitotoxic effects of NMDA and kainate. In: Ito U, Fieshi C, Orzi S, Kuroiwa T, Klatzo I, eds. *Maturation Phenomenon in Cerebral Ischemia III. Defensive Mechanisms Versus Apoptosis. Neuronal Recovery and Protection in Cerebral Infarction. Berlin, Heidelberg, New York: Springer-Verlag,* 1999:328. .
25. Hipkiss AR. Carosine, a protective anti-ageing agent. *Int J Biochem Cell Biol* 1998;30:863–868.

Address reprint requests to:
Prof. A.A. Boldyrev,
Department of Biochemistry
School of Biology
Moscow State University
Vorobjovy Gory
119899 Moscow
Russia

E-mail: aab@1.biocenter.bio.msu.ru