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Thiolic antioxidant supplementation of the diet reverses age-related behavioural dysfunction in prematurely ageing mice

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Abstract

We have studied in a model of premature ageing in mice based on their impaired behavioural response in a simple T-maze test the effect of the ingestion of thioproline (TP) plus *N*-acetylcysteine (NAC) (0.1% w/w of each antioxidant) by female and male mice of Swiss and BALB/c strains on performance in two behaviour tests. The antioxidant treatment (4 weeks in two different periods of life, i.e., adult and old age) protected all animals against early-age-associated behavioural impairment, but this improvement was more evident in the prematurely ageing mice (PAM) in comparison to the control group or non-prematurely ageing mice (NPAM). An improvement of the exploratory activity and neuromuscular coordination after the thiolic antioxidant treatment was found in the PAM, bringing the behavioural parameters to the NPAM levels. These effects could be due to the glutathione precursor role of NAC and TP that replenish the intracellular reduced glutathione (GSH) levels despite advancing age. In conclusion, diet supplementation with thiolic compounds appears to be an effective therapy for protection against early behavioural decline in prematurely ageing mice.

Keywords: Thioproline; *N*-acetylcysteine; Exploratory activity; Neuromuscular coordination; Ageing

1. Introduction

It is well known that an age-related brain function decline may occur even in the absence of neurodegenerative disease (Busciglio et al., 1998; Shukitt-Hale, 1999; Brewer, 2000) because of the attack of reactive oxygen species (ROS) arising from either increased formation of ROS, impaired ability of the aged brain to cope with oxidative stress, or both (Reiter, 1995; Choi and Yu, 1995). The role of oxidative stress is significant especially in the brain, which consumes 20% of all inspired oxygen, contains relatively low concentrations of antioxidants, and has abundant polyunsaturated fatty acids that serve as major biological targets for the ROS (Halliwell, 1992; Rice-Evans and Burdon, 1993).

Antioxidants are essential for protection of the cells against the damaging action of free radicals. Especially, reduced glutathione (GSH) is considered an important and efficient antioxidant mechanism that maintains cells in a reduced condition and protects the organs and tissues against oxidative damage (Meister and Anderson, 1983). GSH is a major brain protector against oxidative stress by interacting directly with ROS or by participating in enzyme-catalyzed redox cycling reactions (Cooper and Kristal, 1997). After scavenging ROS, GSH is transformed into oxidized glutathione (GSSG), which is regenerated to GSH by enzymatic or nonenzymatic reactions. The concentration of GSH in brain is much higher than in blood or cerebrospinal fluid. Therefore, the brain must have a very efficient system to accumulate GSH or, more likely, to synthesize it in situ (Cooper and Kristal, 1997).

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Several studies have shown that the levels of GSH and of total glutathione (GSH plus GSSG) in the rodent brain decline with age and that the brains of older rodents are more susceptible to peroxidative stress (reviewed by Benzi and Moretti, 1995). In agreement with the above, diet supplementation with thiolic compounds that act as glutathione precursors, i.e., l-thiazolidine-4-carboxylic acid [thioproline (TP)] and *N*-acetylcysteine (NAC), could have favorable effects replenishing the glutathione levels, and thus protecting against the age-related decline of behaviour. Accordingly, we have carried out a longitudinal study in a prematurely ageing mouse model. Mice that take a longer time to complete the exploration of the first arm of a T-shaped maze [prematurely ageing mice (PAM)] show early alterations in several nervous functions (behavioural and monoaminergic parameters) (Viveros et al., 2001; De la Fuente et al., 2003), which results in a shorter life span in comparison to those which quickly explore the maze [non-prematurely ageing mice (NPAM)]. These studies demonstrate that, at the same chronological age, the PAM, which show an age-related inability to cope with stress, as well as premature alterations of different nervous and immune functions, are "biologically older" than the control group or NPAM (Guayerbas et al., 2000, 2002b; Guayerbas and De la Fuente, 2003).

In the present longitudinal work, we have investigated the beneficial effect of a diet supplemented with TP 0.1% w/w plus NAC 0.1% w/w on two behavioural tests to which the PAM are subjected, namely, the exploratory activity T-maze test and the neuromuscular coordination test (Miquel and Blasco, 1978).

2. Materials and methods

2.1. Animals and experimental procedure

We have used mice of the ICR (CD-1, Swiss) strain (90 females and 50 males) and of the BALB/c strain (60 females and 40 males) (Harlan Interfauna Ibérica, Barcelona, Spain), which were 10-week old on arrival to our laboratory. Mice were specific pathogen-free as tested by Harlan according to FELASA recommendations. They were randomly divided in groups of five mice, and each group was housed in polyurethane boxes at a constant temperature (22±2 °C) in sterile conditions inside an aseptic air negative-pressure environmental cabinet (Flufrance, Cachan France) on a 12:12-h reversed light/dark cycle. All animals were fed water ad libitum. The diet was in accordance with the recommendations of the American Institute of Nutrition for Laboratory Animals. Mice were treated according to the guidelines of the European Community Council Directives 86/6091 EEC.

At 12 weeks of age, the spontaneous exploratory behaviour of each mouse was tested in a T-shaped maze (with arms 25 cm in length). The test is performed holding the mouse from the tip of the tail and placing it inside the "vertical" arm of the maze with its head facing the end wall. The performance is evaluated by determining with a chronometer the time elapsed until the animal crosses with both hindlegs the intersection of the three arms. This test was performed four times, once every 15 days, to sort out the prematurely ageing mice (PAM; which required over 20 s to complete the exploration of the first arm of the maze, from the non-prematurely ageing mice (NPAM) that completed that exploration in 20 s or less. Animals showing an intermediate response in the T-maze were removed from the study.

Then, each group was subdivided into a control and a treated population. The treated animals received a diet supplemented with 0.1% w/w of both thioproline and *N*-acetylcysteine (NAC) for 4 weeks at adult age (from 29 to 32 weeks old in the Swiss strain; from 32 to 35 weeks old in the BALB/c strain) and for 4 weeks at old age (from 75 to 78 weeks old in the Swiss strain; from 56 to 59 weeks old in the BALB/c strain). Control groups were fed standard food. Thus, we have four groups for each strain and sex, i.e., PAM controls (PAMC), NPAM controls (NPAMC), PAM fed antioxidants (PAMA), and NPAM fed antioxidants (NPAMA).

2.2. Neuromuscular coordination and vigor test (tightrope test)

In the tightrope test (Miquel and Blasco, 1978), the mouse is placed on the middle of a tightrope tied up on each side to the rod of a chemical stand. The tightrope, which is 60 cm in length, is suspended above a mouse cage at about 40 cm of its bedding of wood shavings. The mice have to reach one of the side poles. Only 1 min is allowed for completion of the test. The time in seconds (until contact with the pole is made by the head, body, or limbs) is recorded, and the animal is scored positive. Failure to reach the pole, either by falling or by not progressing enough towards the sides in the allowed time of 1 min, is scored negative. The data have been shown as the time, in seconds, that the mice completing the test spent in carrying it out.

In this longitudinal study, the exploratory activity and neuromuscular coordination were measured at 29, 33, 46, 55, 64, and 79 weeks of age in the Swiss strain and at 32, 36, 44, 54, 60, 72, and 80 weeks of age in the BALB/c strain. The data from mice that spent 60 s or more to perform the tests were not included in the results.

2.3. Statistical analysis

The data were considered to be nonparametric according to the Kolmogorov–Smirnov test and are expressed as the median of the time in seconds. The values range from 3 to 58 s in both behavioural tests. The Friedman test, for differences among groups, and the Mann–Whitney *U* test,

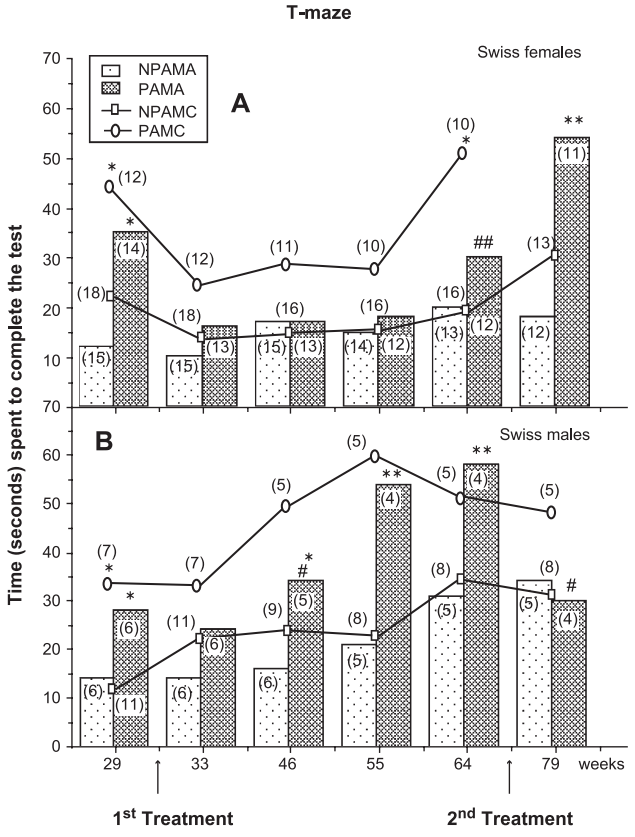

Fig. 1. Each column represents the median of the time (s) spent to carry out the T-maze test for the surviving animals at each time (in brackets). (A) Swiss females; (B) Swiss males. *p<0.05, **p<0.01 differences between NPAM and PAM; # p<0.05, ##p<0.01 with respect to the corresponding control group (represented by line). NPAMA—non-prematurely ageing mice treated with antioxidants; NPAMC—non-prematurely ageing mice treated with control diet; PAMA—prematurely ageing mice treated with antioxidants; PAMC—prematurely ageing mice treated with control diet.

for comparison between each two groups, from SPSS software were used, with p<0.05 being the minimum significance level.

3. Results

As shown in Fig. 1A, at 29 weeks of age, when the mice were fed a control diet, the female Swiss PAM spent significantly more time to carry out the T-maze test than the NPAM (p<0.05). After 4 weeks on a diet supplemented with 0.1% NAC plus 0.1% TP (from 29 to 32 weeks of age), the time employed by the PAMA group to perform the test was decreased with respect to the control group (PAMC) of the same age, although the difference did not reach a significance level. This decrease was maintained for successive weeks, although it only reached the significance level at 64 weeks of age (p<0.01) with respect to the values of PAMC of the same age (represented by a line).

The second antioxidant treatment of aged mice, although resulted in a higher time to perform the test than that observed at younger age, showed a positive effect since a number of PAMA performed successfully the T-maze test at 79 weeks of age, whereas, in the control group, no animals were able to perform the test.

The results of the exploratory activity test obtained on male Swiss mice are shown in Fig. 1B. At 29 weeks of age, the PAM group took a longer time to perform successfully the T-maze test than the NPAM (p<0.05) group. The first treatment in the adult age did not influence significantly the exploratory activity of the NPAMA, although the values found in the treated mice remained lower than those of the controls of the same age (as shown by a line). However, the PAMA took a shorter time to perform the test than the PAMC, with a statistically significant difference at 46 (p<0.05) weeks of age. With advancing age, the time required to complete this behavioural test increased significantly, mainly in the PAM group. Otherwise, the second antioxidant treatment (from 75 to 78 weeks of age)

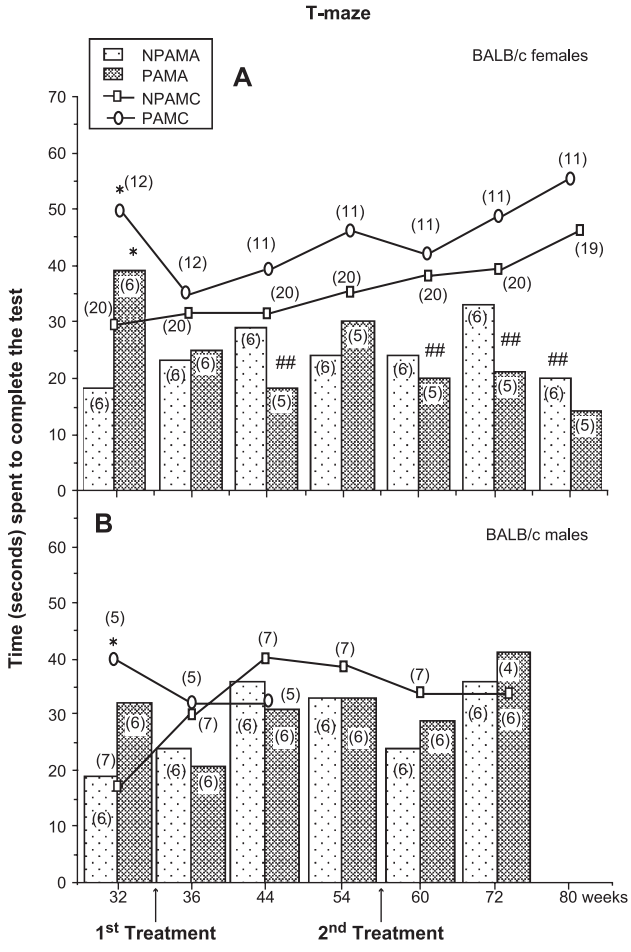

Fig. 2. Each column represents the median of the time (s) spent to carry out the T-maze test for the surviving animals at each time (in brackets). (A) BALB/c females; (B) BALB/c males. *p<0.05 differences between NPAM and PAM; # p<0.05, ##p<0.01 with respect to the corresponding control group (represented by a line). NPAMA—non-prematurely ageing mice treated with antioxidants; NPAMC—non-prematurely ageing mice treated with control diet; PAMA—prematurely ageing mice treated with antioxidants; PAMC—prematurely ageing mice treated with control diet.

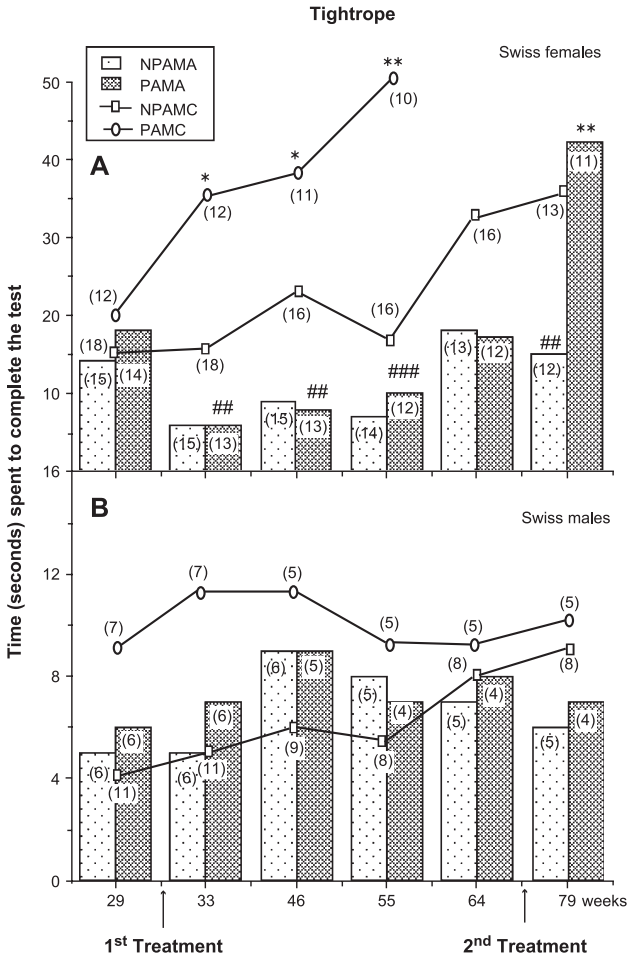

Fig. 3. Each column represents the median of the time (s) spent to carry out the tightrope test for the surviving animals at each time (in brackets). (A) Swiss females; (B) Swiss males. *p<0.05, **p<0.01 differences between NPAM and PAM; ##p<0.01, ###p<0.001 with respect to the corresponding control group (represented by line). NPAMA—non-prematurely ageing mice treated with antioxidants; NPAMC—non-prematurely ageing mice treated with control diet; PAMA—prematurely ageing mice treated with antioxidants; PAMC—prematurely ageing mice treated with control diet.

improved the exploratory activity of the PAMA group, reducing the time needed to perform the behavioural test, and maintained it significantly lower with respect to the controls of the same age (p<0.05).

At 32 weeks of age, in the female-BALB/c-mice-fed control diet (Fig. 2A), the time spent to carry out the T-maze test was higher in the PAM than in the NPAM (p<0.05). After the first treatment (from 32 to 35 weeks) with 0.1% NAC plus 0.1% TP, the exploratory activity of the PAMA improved with respect to the PAMC (p=0.05 at 44 weeks of age). The treatment allowed the NPAMA to always maintain the values of the test below those of the NPAMC of the same age (as shown in the figure by a line). The second antioxidant treatment (from 56 to 59 weeks) improved this capacity in the PAMA group, in such a way that the time needed to complete the test was decreased with respect to the PAMC of the same age (p<0.01). A similar effect was obtained in the NPAMA at 80 weeks of age.

In male BALB/c mice (Fig. 2B), the differences between PAM and NPAM, determined at 32 weeks of age, were statistically significant in the control group (p<0.05). The effect of the first antioxidant treatment was not significant, although the values were lower than those of the respective controls (represented by a line), although the difference did not reach statistical significance. It should be taken into consideration that the PAMC did not perform the T-maze test after 44 weeks of age. The second treatment at old age (56–59 weeks) allowed to maintain this capacity up to 72 weeks of age.

As regards the tightrope test, the result obtained on female Swiss mice (Fig. 3A), expressed as the time spent to perform successfully this behavioural test, showed an improvement after the 0.1% NAC plus 0.1% TP diet supplementation, although with statistical significance only in the PAMA (p<0.01). This improvement was maintained until 55 weeks of age, as shown by the lower values with respect to the corresponding controls (p<0.001). The

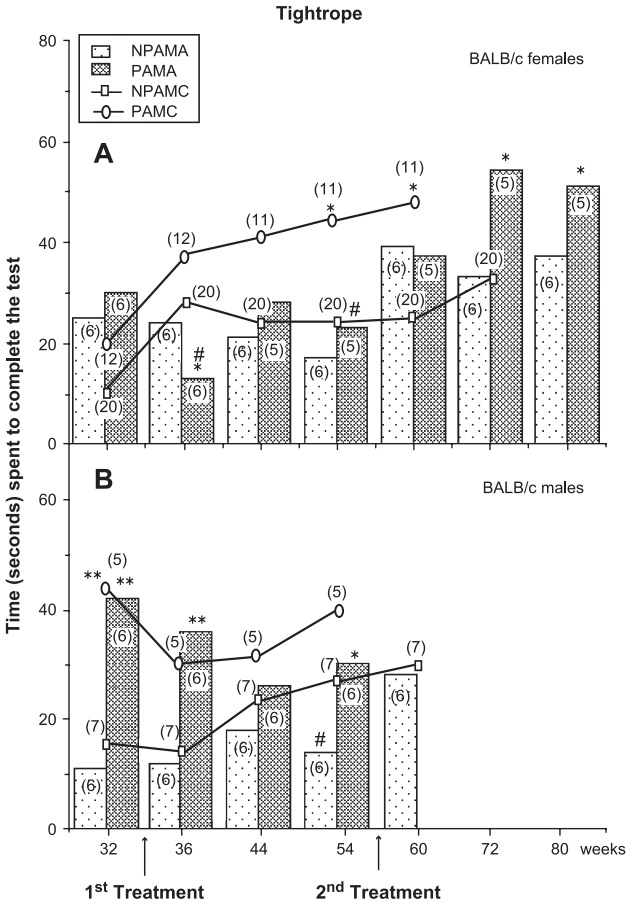

Fig. 4. Each column represents the median of the time (s) spent to carry out the tightrope test for the surviving animals at each time (in brackets). (A) BALB/c females; (B) BALB/c males. *p<0.05, **p<0.01 differences between NPAM and PAM; #p<0.05 WITH respect to the corresponding control group (represented by line). NPAMA—non-prematurely ageing mice treated with antioxidants; NPAMC—non-prematurely ageing mice treated with control diet; PAMA—prematurely ageing mice treated with antioxidants; PAMC—prematurely ageing mice treated with control diet.

PAMC did not carry out successfully the tightrope test since 64 weeks of age onward, and, after the second antioxidant treatment, the treated PAM could perform it up to 79 weeks. The time employed by the NPAMA to perform the test was also improved ($p<0.01$).

In male Swiss mice (Fig. 3B), the differences between 29-week-old NPAM and PAM did not reach the significance level, and the antioxidant treatments in adult and old age did not show significant effect, only trends.

Fig. 4A shows the results for females BALB/c mice. For 32-week-old mice, there were no significant differences between NPAM and PAM in the time employed to perform the tightrope test. The first antioxidant treatment reduced significantly this time in the PAMA ($p=0.04$), showing this effect up to 54 weeks of age. The PAMC, at the two last ages, and the NPAMC at 80 weeks of age did not perform the tightrope test. However, after a second treatment, they became able to perform successfully the tightrope test.

As regards the male BALB/c mice (Fig. 4B), the differences between 32-week-old NPAM and PAM were statistically significant, with the PAM showing higher values than the NPAM ($p<0.01$). The first NAC plus TP treatment only showed effect on the neuromuscular coordination test at 54 weeks of age in the NPAMA ($p<0.05$).

4. Discussion

A low performance in neuromuscular coordination and exploration tests is considered a marker of neurological ageing (Forster et al., 1996). Furthermore, recent studies suggest that oxidative damage due to free radicals may be an important factor in the age-related impairment of the central nervous system (CNS) (Choi and Yu, 1995; Knight, 1997; Brewer, 2000). Therefore, a preventive approach to protect this system against senescent ROS injury is to increase its antioxidative defences by dietary antioxidant supplementation.

In agreement with the above, our present data show that a dietary administration of the thiolic antioxidants TP and NAC in two different periods of the life of mice protects against age-related behavioural impairment, especially in the PAM. These, as it has been observed in Swiss mice, suffer a premature ageing process as shown by their decreased locomotor and exploratory activity in stressful situations, a less adaptive response to stress in the hole-board, the open field and the plus-maze tests (Viveros et al., 2001), a worse neuromuscular coordination (Guayerbas et al., 2002a), and a deficient monoaminergic system in several brain regions (De la Fuente et al., 2003) in comparison to the NPAM. In addition, the PAM, in both the Swiss and BALB/c strains, show an impairment of immune functions in comparison to the NPAM (Guayerbas et al., 2002b; Guayerbas and De la Fuente, 2003). These premature senescent changes are probably linked to the shorter life span of the PAM as compared to the NPAM. Moreover, the better performance in the T-maze of BALB/c females than Swiss females is related to the greater longevity of the former (Guayerbas and De la Fuente, 2003).

Recently, it has been indicated that the poor performance in the above behavioural tests in our mouse model of premature ageing is due to a higher brain oxidation stress as compared to that suffered by NPAM (Navarro et al., 2002). They observed that the worst performance in the tightrope and exploratory activity tests is accompanied by an increased lipid peroxidation and changes in the activities of several mitochondrial enzymes in the brain.

Our present data show that the above behavioural deficits of the PAM as regards exploratory activity in the T-maze and neuromuscular coordination can be prevented by thiolic antioxidants. Thus, diet supplementation with TP plus NAC improved the exploratory activity in the T-maze, decreasing the time consumed to perform this test. The old-PAM-fed unsupplemented diet was unable to carry out successfully the behavioural tests. Nevertheless, they became able after antioxidant therapy. The greatest improvement was found in BALB/c females in the T-maze, with the animals responding to the antioxidant administration as early as at 44 weeks of age. This fact could be related to the increased life span observed in this sex and strain after the same supplementation with antioxidants (data in the publication process). Moreover, the neuromuscular coordination evaluated by the tightrope test showed an improvement after the NAC plus TP treatment, which was more evident in female mice. The Swiss males of the same age showed a good preservation of their motor behaviour even when fed diet was not supplemented with these thiolic antioxidants. This fact was not observed in BALB/c mice, which reveals an interesting difference between the two strains. Accordingly, the activity of the bc1-complex in skeletal muscle mitochondria declines sharply in females, but not in males, with age (Boffoli et al., 1996).

Our present data are in agreement with other studies showing the effectiveness of chronic antioxidant supplementation for reversing or alleviating the injurious action of ROS on CNS functions (Sastre et al., 2000; Bondy et al., 2002; Topic et al., 2002). The mechanisms involved in the favourable effects of these thiolic antioxidants are not well understood, but they may be linked to the free radical-scavenging effect resulting from the direct reaction between the reducing thiol groups of NAC and TP and ROS. Furthermore, these effects are probably linked to the increase in the intracellular glutathione concentration caused by NAC and TP, which reverses the oxidation responsible for the age-associated cell dysfunctions (Dröge, 2002). In agreement with this hypothesis, recent data from our laboratory demonstrate an increase in the glutathione levels in the brain of PAM after a 5-week NAC plus TP diet supplementation (unpublished data). Other authors have observed a beneficial effect of chronic NAC

administration on age-related memory impairment and passive avoidance behaviour in mice (Martínez et al., 1997, 2000), an effect that according to these authors is unrelated to the NAC-induced increase in mitochondrial glutathione. Therefore, NAC would probably act on the mitochondrial oxidative phosphorylation complexes, protecting them against oxidative damage by preserving the protein sulfhydryl groups and/or protecting the mitochondrial membranes against the lipid peroxidation that can impair mitochondrial complex activities. On the other hand, the work from García de la Asunción et al. (1996) and the more recent data reviewed by Sastre et al. (2000) indicate that the oxidative damage to the mitochondrial genome that takes place with age in the liver, kidney, and brain of rats is directly related to the oxidation of the mitochondrial glutathione. This highlights the key role of an adequate supply of dietary thiolic antioxidants (such as NAC and TP) to protect the intramitochondrial levels of glutathione and therefore of mtDNA since, as emphasized by Miquel (2002), ATP biosynthesis and physiological performance depend on the integrity of the mitochondrial genome.

In relation to the above and with focus on the immune system functions (the impairment of which most likely plays a central role in the short life span of our PAM), it may be of interest that, on the one hand, mutations in mtDNA occur in peripheral blood lymphocytes, which may result in reduced responsiveness to antigens and increased apoptosis (Drouet et al., 1999), and, on the other hand, we have observed good preservation of mitochondrial structure in lymphocyte cultures from aged mice in medium supplemented with TP (De la Fuente, unpublished finding).

As regards the antiageing usefulness of other thiolic antioxidants, Shukitt-Hale et al. (1999) found that diet supplementation with GSH improved the motor performance in an inclined screen test of 24-month-old mice. They supplemented the diet with other antioxidants such as vitamin E, melatonin, and strawberry extract, but only the glutathione-enriched diet showed a beneficial effect in that test. Furthermore, another thiolic compound, namely, DL-a-lipoic acid (LA), has been shown to act as a potent antioxidant by inhibiting lipid peroxidation and regenerating other antioxidants in the brain of aged rats (Arivazhagan et al., 2002). According to the recent review by Miquel (2002) and the experimental data of Liu et al. (2002) obtained on LA-administered old rats, the beneficial effects of this thiolic antioxidant against the ageing process would be especially important at the mitochondrial level.

In our opinion, the above data justify the concept that a supplementation of the diet with TP plus NAC may be useful to protect, not only against age-related immune dysfunction (Blanco et al., 1999; De la Fuente et al., 2002), but also to preserve behavioural competence in senescent animals. Further clinical research will be needed to find out if diet supplementation with thiolic antioxidants may be useful to protect brain function in elderly humans.

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