The Antioxidant N-acetylcysteine In Vitro Improves Several Functions of Peritoneal Leucocytes from Old Mice Approaching their Values to those of Adult Animals

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Summary

The age-related deterioration of the function of immune cells, or immunesenescence, is based on oxidative stress (imbalance between the levels of oxidants and antioxidant defences with an increase of the first). Accordingly, the ingestion of a diet supplemented with thiolic antioxidants such as N-acetylcysteine (NAC), a glutathione precursor, by aged subjects improved their leucocyte functions. The aim of the present study was to show if NAC improves *in vitro* several functions of leucocytes from chronologically old mice and if this antioxidant is able to bring the values of these functions to the levels of those of adult animals. Six concentrations of NAC (in a range from 0.001 mM to 2.5 mM) were investigated on several functions of peritoneal leucocytes from old (78±2 weeks of age) BALB/c mice. These functions were those of the phagocytic process in macrophages, namely adherence to substrate, directed migration or chemotaxis, phagocytosis of inert particles and superoxide anion levels as a measure of digestion capacity, as well as of adherence and chemotaxis of lymphocytes. These functions were also studied in peritoneal leucocytes from adult (18±2 weeks of age) mice. The results showed that NAC *in vitro* improves all the functions studied, especially at the highest concentrations, which had shown impaired values in old mice, approaching those of adult animals. Since the immune functions studied are markers of health and predictors of longevity, the administration to aged subjects of NAC, which shows a direct action in leucocytes, seems to be a good strategy to improve their immune system and, therefore, to reach a healthy longevity.

Key Words: N-acetylcysteine; immunosenescence; macrophages; lymphocytes; mice

Running Title: N-acetylcysteine and leucocytes from old mice

INTRODUCTION

Ageing is accompanied by an impairment of the physiological systems including the immune system. In fact, it is well known that with the passage of time there is a decrease in the resistance to infections and an increase in autoimmune processes and cancer. This indicates the presence of a less competent immune system, which exerts a great influence on the increasing morbidity and mortality observed in ageing human subjects (Wayne et al. 1990). Moreover, the high death rate found in aged populations is due in great proportion to infectious processes (High 2004). Thus, it is presently accepted that almost every component of the immune system undergoes striking age-associated re-structuring, leading to changes that may include enhanced as well as diminished functions, this fact being denominated immunosenescence (Aw et al. 2007, De la Fuente and Miquel 2009). We have studied the changes in several immune cell functions through age of experimental animals, such as rats and specially mice, and of human beings, and we have observed a similar age-related evolution of many of these functions in immune cells from peripheral blood of humans and from the peritoneum of mice (De la Fuente 2002, De la Fuente et al., 2004a,b,2005; De la Fuente and Miquel 2009, Arranz et al., 2010; De la Fuente et al. 2011a). In agreement with the oxidation theory of ageing (Harman 1957, Miquel et al. 1980, Miquel 1998), we have observed that those age-related changes of immune cell functions have as their basis an oxidative and inflammatory stress situation, which has among its intracellular mechanisms the activation of the NFkB in the immune cells (De la Fuente et al. 2005, De la Fuente and Miquel 2009, Arranz et al. 2010). Moreover, we have proposed a key involvement of the immune system in the rate of ageing of each organism, since there is a relation between the redox state and functional capacity of the immune cells and the longevity of individuals (De la Fuente and Miquel 2009, Alonso-Fernandez and De la Fuente 2011). A confirmation of this role of the immune system in the oxi-inflamm-ageing is that the administration of adequate amounts of antioxidants in the diet, which improves the immune cell functions, decreasing their oxidative stress, in experimental animals and humans, increases the longevity of mice (De la Fuente et al. 2008, Arranz et al. 2008, De la Fuente and Miquel 2009, De la Fuente 2010, De la Fuente et al. 2011a,b).

In this context, we have especially studied the effects of diet supplemented with thiol antioxidants such as n-acetylcysteine (NAC). This antioxidant neutralizes free radicals in a direct manner (Gressier et al. 1994) and is a glutathione precursor (De

Flora et la. 1991). Glutathione (GSH) is the principal nonenzymatic antioxidant of the cells and plays a major role in preservation of an adequate intracellular redox state (Dröge 2002). Moreover, an optimal immune response will require adequate levels of GSH (Dröge and Breikreuz 2000). With ageing, there is a decrease in this GSH content, which has been observed in a variety of cells and tissues, including those of the immune system (Hernanz et al. 2000, Dröge 2005, Arranz et al. 2008). NAC has been studied by many authors because of its wide range of effects at all cellular levels and with multiple clinical applications (Dodd et al. 2008, Millea 2009). In previous studies we have observed that the ingestion of a diet supplemented with NAC improves many leucocyte functions in adult prematurely ageing mice (Puerto et al. 2002, Guayerbas et al. 2005). In addition, in postmenopausal women NAC showed similar effects (Arranz et al. 2008). The ingestion of a diet supplemented with NAC and other thiolic precursors namely thioproline, also improved many leucocyte functions in adult, prematurely ageing mice and old animals (Blanco et al. 1999, De la Fuente et al. 2002, Guayerbas et al., 2002b,2004, De la Fuente 2010). NAC *in vitro* stimulated several functions of lymphocytes and macrophages from adult mice (Del Rio et al. 1998, Pomaki et al. 2005,De la Fuente et al. 2011b) and human subjects (Karlsson et al., 2011). However, the effects *in vitro* of NAC on functions of peritoneal leucocytes from old mice have not been studied yet. Moreover, since the amount of thiol antioxidants in the diet that improve immune function depends on the age of the animals (De la Fuente et al. 2002), and based on the above, the aim of the present work was to test the effects of a wide range of concentrations of NAC *in vitro* on several functions of peritoneal macrophages and lymphocytes from old mice, and to check if the NAC administration approaches the values obtained to those in adult mice.

MATERIAL AND METHODS

Animals

Old (78±2 weeks of age) and adult (18±2 weeks of age) female BALB/c mice (*Mus musculus*) (Iffa Credo, France) were used. The mice were specific pathogen free, as tested by Harlan according to FELASA recommendations. Twelve mice of each age were used. They were randomly divided in groups of 6, and each group was housed in a polyurethane box, at a constant temperature (22 ± 2º C) in sterile conditions inside an aseptic air negative-pressure environmental cabinet (Flufrance, Cachan, France), on a

12/12h reversed light/dark cycle. All animals were fed water and standard Sander Mus (A.04 diet from Panlab L.S. Barcelona, Spain) pellets *ad libitum*. The diet was in accordance with the recommendations of the American Institute of Nutrition for laboratory animals. The experimental protocol was approved by the Animal Ethics Committee of the Complutense University of Madrid (Spain).

Collection of peritoneal leucocytes

After being housed for 2 weeks, mice were sacrificed by cervical dislocation according to the guidelines of the European Community Council Directives 86/6091 EEC, between 8:00 and 10.00 h. The abdomen was cleansed with 70% of ethanol, the abdominal skin was carefully dissected without opening the peritoneum and 4 ml of sterile Hank's solution was injected intraperitoneally. Then, the abdomen was gently massaged and peritoneal resident cells were removed, allowing the recovery of 85-90% of the injected volume. Macrophages, identified by morphology and non-specific esterase staining, were counted in Neubauer chambers and then adjusted by dilution with Hank's solution to $5x10^5$ macrophages/ml. Lymphocytes were also identified by morphology and adjusted to $5x10^5$ lymphocytes/ml Hank's medium. The cellular viability, determined in each experiment using the trypan-blue exclusion test, was in all cases higher than 95%.

Antioxidant

N-acetylcysteine (NAC) was purchased from Sigma (St Louis, MO, USA) and the following concentrations were used: 0.001mM, 0.01 mM, 0.1 mM, 1 mM and 2.5 mM dissolved in Hank´s solution.

Assays of phagocytic function in peritoneal macrophages

In the peritoneal suspension, with macrophages adjusted to $5x10^5$ cells/ml Hank's solution, we carried out the study of the different steps of the phagocytic process, i.e., adherence to tissues, mobility to infectious focus (chemotaxis), phagocytosis of foreign inert material and digestion capacity of this material through the production of intracellular free radicals, namely the superoxide anion, which is the first response in the respiratory burst.

For the quantification of adherence capacity to the substrate, we observed the adherence to a smooth plastic surface, because it resembles adherence to animal tissue.

The method was carried out as previously described (Puerto et al. 2002). Briefly, aliquots of 0.2 ml of the peritoneal suspensions were placed in eppendorf tubes and incubated 10, 20 and 30 min at 37ºC, and after gently shaking the number of non-adhered macrophages was determined in Neubauer chambers. The adherence index, AI, was calculated according to the following equation: AI= (Mi–Mf /Mi) x 100, where Mi is the initial concentration of macrophages ($5x10^5$ cells/ml) and Mf the final concentration of macrophages in the supernatant (non-adherent cells) after each incubation time.

The chemotaxis assays were performed according to a modification (Puerto et al. 2002) of the original technique described by Boyden (1962), which consists basically in the use of chambers with two compartments separated by a filter (Millipore, Bedford, MA) with a pore diameter of 3 nm. Aliquots of 0.3 ml of the peritoneal suspension were deposited in the upper compartment of the Boyden chambers. F-met-leu-phe (Sigma, St. Louis, MO) (a positive chemotactic peptide in vitro), at 10^{-8}M, was placed in the lower compartment in order to determine chemotaxis. The chambers were incubated for 3h at 37ºC and 5% CO_2, and after this time the filters were fixed, stained and the chemotaxis index (C.I.) was determined by counting in an optical microscope (immersion objective) the total number of macrophages in one third of the lower face of the filters.

The latex phagocytosis assay was carried out following a method previously described (Puerto et al. 2002). Aliquots of 0.2 ml of peritoneal suspensions were incubated in culture plates (Sterilin, Teddington, England) for 30 min. To the adherent monolayer, after being washed with PBS (phosphate buffer saline), 0.02 ml latex beads (1.09 mm diluted to 1 % PBS, Sigma, St.Louis, MO) were added. After 30 min of incubation, the plates were washed, fixed and stained and the number of particles ingested by 100 macrophages was counted, being expressed as phagocytic index (P.I.). Moreover, the percentage of macrophages with phagocytic capacity (ingesting at least one particle) was also counted and expressed as phagocytic efficiency (P.E.).

Superoxide anion production was evaluated assessing the capacity of this anion, produced by macrophages, to reduce nitroblue tetrazolium (NBT). This was carried out following the method described by De la Fuente (1985) slightly modified as follows. Aliquots of 0.25 ml of peritoneal suspension were mixed with 250 ml of NBT (1mg/ml in PBS, Sigma), 0.05 ml of a latex bead suspension were added to the stimulated samples and 0.05 ml of PBS to the non-stimulated samples. After 60 min of incubation, the reaction was stopped, the samples were centrifuged, and the intracellular reduced

NBT was extracted with dioxan (Sigma) and, after centrifugation, the supernatant absorbance at 525 nm was determined. The results were expressed as nmol/10^6 cells using a pattern curve.

Assays of peritoneal lymphocyte functions

The two functions studied in macrophages that are also carried out by lymphocytes, namely adherence and chemotaxis, were studied in these cells of peritoneal suspension adjusted to $5x10^5$ lymphocytes/ml Hank's médium, following similar methods to those described for macrophages.

Statistical analysis

The data are expressed as the mean ±S.D of 10 values corresponding to the same number of experiments. Each value is the mean of the data from an assay performed in duplicate. The data were examined statistically by one-way analysis of variance (ANOVA) for paired observations (NAC effects in the aged mice), followed by the Scheffer´s F post hoc procedure. The ANOVA test for unpaired observations was used for comparing adult and aged mice, followed by the Scheffe ´s F test. The normality of the samples was confirmed by the Kolmogorov-Smirnov test. We used the significance level $2\alpha = 0.05$.

RESULTS

The percentages of macrophages and lymphocytes obtained in the peritoneal suspensions from adult and old mice were 39±11 and 30±13 for macrophages and 61±11 and 70±13 for lymphocytes, respectively.

The adherence and chemotaxis capacities of macrophages are shown in Figure 1. The adherence indexes (Fig. 1A) at 10, 20 and 30 minutes of incubation in macrophages from old mice were higher (statistically significant) than those in cells from adult animals. The presence of NAC decreased the adherence indexes, the differences being statistically significant at 10 and 20 minutes of incubation with 0.1 mM, 1 mM and 2.5 mM. Thus, the adherence indexes with 2.5 mM (at 10 min of incubation) and with 1mM (at 10 and 20 min of incubation) were similar to those in adult mice. The chemotaxis indexes (Fig. 1B) in macrophages from old mice, which in controls were lower (statistically significant) than those in adult animals, increased in the presence of NAC.

This antioxidant from 0.01 to 2,5 mM stimulated the chemotaxis, showing statistically significant differences with respect to the values of old controls. However, this increase did not reach indexes similar to those in adult mice, since in all cases the values were significantly lower than in adult animals.

The results of phagocytosis capacity, both phagocytic index (P.I.) and phagocytic efficiency (P.E) (Fig. 2), show decreased values (statistically significant) in macrophages from old mice in comparison to those in adult animals. NAC increased the P.I. (Fig. 2A) in macrophages from old mice (with the exception of the concentration of 2.5 mM), showing statistically significant differences with the concentrations of 0.01, 0.1 and 1 mM. The levels of IP with the highest concentration of 2.5 mM were significantly lower than those with 1 and 0.1 and 0.01 mM of NAC. The values of IP with all concentrations of NAC were significantly lower than those in adult mice. With respect to the PE (Fig. 2B) the concentration of 1 mM of NAC was the only one that significantly increased this index, however these values did not reach those of macrophages from adult animals.

The levels of superoxide anion, both basal and stimulated (Fig. 3) were significantly increased in cells from old mice with respect to those from adult animals. However, the concentrations of 0.1 and 1 mM of NAC significantly increased these levels in both basal and stimulated samples.

The adherence and chemotaxis capacities of lymphocytes are shown in Fig. 4. The adherence indexes (Fig. 4A) were higher (statistically significant) in lymphocytes from old mice than in those from adult animals at 10 min of incubation. NAC significantly decreased the adherence indexes at this time of incubation with 0.1 mM, 1 mM and 2.5 mM. With 1 mM of NAC the values at 10 min of incubation were similar to those in lymphocytes from adult mice. The chemotaxis of lymphocytes (Fig. 4B) was significantly decreased in cells from old mice in comparison with those from adult animals. No statistically significant differences were found in the presence of NAC.

DISCUSSION

NAC *in vitro* improved several functions of peritoneal macrophages and lymphocytes from old mice, which showed values quite similar to those in adult animals. This fact suggests that the positive effects on these immune functions caused by diet supplementation with this antioxidant in adult prematurely ageing mice

(Guayerbas et al. 2005) are, at least in part, due to a direct action on the immune cells. The results obtained in the present study corroborate that the deterioration of immune cells with ageing is linked to oxygen stress and that the administration of adequate amounts of the antioxidant NAC preserves an appropriate function of the immune cells in the ageing process (De la Fuente et al. 2005, 2011a, De la Fuente and Miquel 2009).

The functions of macrophages studied in the present work are the consecutive steps of the phagocytic process, which is carried out by phagocytic cells in their defensive activity against infections. In this process the first steps involves the adherence of cells to tissue substrate, which is followed by the migration of these cells to the focus of the infection through a chemical gradient (chemotaxis). With ageing peritoneal macrophages increase adherence capacity and decrease chemotaxis (De la Fuente et al. 2004b, De la Fuente and Miquel 2009, Arranz et al. 2010). These changes have also been observed in the present study and show the oxidative stress that old mice suffer. This is related to the increase of adhesion molecules involving activation of NFkB (Lavie et al. 2005, Victor et al. 2005, Arranz et al. 2010). An oxidative stress situation is also linked to a release of the migration inhibitor factor (MIF) (Hirokawa et al. 1998, Victor et al. 2005), which could explain the decrease of chemotaxis in leucocytes from old mice.

NAC decreases the adherence capacity in macrophages from old mice at 0.1 mM. 1 mM and 2.5 mM, especially at 10 and 20 min of incubation, with the values of this function being more similar to those in adults. When we studied the effects of NAC (0.1 to 5 mM) *in vitro* on the adherence of macrophages from adult mice, no change (Del Rio et al. 1998, Puerto et al. 2002,Pomaki et al. 2005) or an increase (Victor and De la Fuente 2002) were found. Thus, NAC modulates adherence of macrophages, no affecting or increasing this function when cells are from adult animals, but decreasing it in macrophages from old mice, in which the oxidative stress situation of the animals is shown with an increased adherence (De la Fuente et al. 2005, De la Fuente and Miquel 2009). Similar results, namely a decrease of the peritoneal macrophage adherence capacity in presence of NAC, were obtained in cells from adult mice with lethal endotoxic shock, a situation with an acute oxidative stress that increases the adherence of these phagocytes (Victor and De la Fuente 2002).

Chemotaxis was stimulated by NAC, especially with the highest concentrations. Similar results were obtained in macrophages from adult mice (Del Rio et al. 1998, Puerto et al. 2002, Victor and De la Fuente 2002). Several studies have found a relation

between supplementation and deficiency of antioxidants, and the increase or decrease of chemotaxis, respectively (Bendich 1989,De la Fuente et al. 2000). In addition, NAC administration to postmenopausal women, which showed a decreased chemotaxis of peripheral blood neutrophils, increased this function (Arranz et al. 2008). Moreover, in prematurely ageing mice, with a decreased chemotaxis in the peritoneal macrophages, the administration of diet with NAC increases this function (Puerto et al. 2002, Guayerbas et al. 2005). These effects could be due to a direct action of NAC on phagocytic cells from old subjects, since in the present study we have observed an increase of chemotaxis in presence of NAC *in vitro*. This stimulation of chemotaxis in macrophages from animals with an oxidative stress situation and a decreased chemotaxis was also found in cells from adult mice with a lethal endotoxic shock, in which NAC acted decreasing the high activation of NFkB caused by endotoxin in the immune cells (Victor and De la Fuente 2002,2003, Victor et al. 2005).

An adequate chemotaxis capacity that allows the phagocytes to reach the focus of the infection is followed by the ingestion of the foreign agents. The phagocytic capacity decrease with ageing (De la Fuente et al. 2004b, De la Fuente and Miquel 2009, Arranz et al. 2010), and this fact is also observed in the present work in both activities, the percentage of particles ingested (phagocytic index) and of macrophages ingesting at least a particle (phagocytic efficiency). Previous work showed that peritoneal macrophages use their endogen antioxidants when they are phagocyting (Herranz et al. 1990). This could explain that NAC and other antioxidants *in vitro* increase the phagocytosis of macrophages from adult mice (Del Rio et al. 1998, Puerto et al. 2002, Victor and De la Fuente 2002, Pomaki et al.2005). In macrophages from old mice this effect is shown with 0.1 and 1 mM, but not with 2.5 mM, however, this concentration is effective in macrophages from adult mice (Puerto et al., 2002,Victor and De la Fuente 2002). Previous studies also showed that a higher concentration of a thiol antioxidant produce less effect on phagocytosis than other lower concentrations, as observed with GSH (Del Rio et al. 1998). Moreover, no effect on phagocytosis was found with 5 mM of NAC in macrophages from adult mice, but an increase of this function was found with lower concentrations (Pomaki et al. 2005). Although the amount of thiolic antioxidant such as NAC and thioproline that adult and old animals have to ingest with diet to improve phagocytic function was higher in old mice than in adult animals (De la Fuente et al. 2002), this it is not observed *in vitro*. It is possible that *in vitro* a high concentration of NAC neutralizes so much the ROS produced by the cell,

and this cannot carry out its function adequately. It is known that leucocytes produce ROS as chemical weapons to incapacitate pathogens and malignant cells and as modulators of gene expression, regulating the biosynthesis of many immune mediators (Knight 2000, Yoon et al. 2002). Moreover, macrophages are the immune cells more clearly involved in the oxidant generation, since they use free radicals in order to perform their defensive functions such as the phagocytic activity (De la Fuente 2008, De la Fuente and Miquel 2009). It is possible that another imbalance ROS/antioxidant appear with lower levels of ROS than those that cell needs when there are high concentrations of NAC *in vitro*. In addition, in a recent study moderate concentrations of NAC in vitro (0.4-3.2 mM) increased alloantigen-induced proliferation, expression of activation markers CD25 and CD71 on T cells, and production of IFN-gamma and IL-10, whereas high concentrations of NAC (12.5-50 mM) were suppressive (Karlsson et al. 2011). The results *in vivo* on old subjects commented above suggest that the alterations of the digestive tract and in the passage of the antioxidants through the gut, that appear with ageing, only allow to get adequate levels of these compounds to the immune cells in old animals when the amount of the antioxidant in the diet is high.

In the presence of a phagocytic stimulus, macrophages initiate what is known as the respiratory burst, characterized by the production of free radicals, the first of which is the superoxide anion. The levels of this free radical in phagocytes of old mice increase with respect to that of adult animals (De la Fuente et al. 2002,Guayerbas et al. 2002), although we have found opposite results (De la Fuente et al. 2004, De la Fuente et al. 2011). In previous work we have observed increased levels of intracellular superoxide anion in macrophages from adult mice after *in vitro* treatment with NAC (Del Rio et al. 1998, Victor and De la Fuente 2002). This effect was also found with GSH (Del Rio et al. 1998), an antioxidant that also increases neutrophil oxidative burst activity (Atalay et al. 1996). These results show that the neutralizing capacity of NAC does not interfere with the generation of superoxide anion. Thus, it has been observed that some antioxidants can efficiently neutralize extracellular phagocyte-derived oxidants without affecting the bactericidal oxygen radicals inside the intracellular phagosomes (Jariwalla and Harakeh 1996). Moreover, antioxidants such as ascorbic acid increase the activity of the hexose monophosphate shunt in neutrophils leading to the synthesis of NADPH, which is needed for reduction of molecular oxygen to superoxide anion, as well as increase NBT reduction (Anderson 1979). This fact could explain the increments obtained in both non-stimulated and stimulated cells in presence

of NAC. Moreover, under appropriate conditions, superoxide anion can be generated as a consequence of radical scavenging by thiol antioxidants like GSH in conditions usually found intracellularly. Then, superoxide would act as a radical sink being removed enzymatically by SOD (Winterbourn 1993). In addition, ingestion of a diet supplemented with thioproline and NAC increases the levels of superoxide anion (De la Fuente et al. 2002) or preserves these levels (Blanco et al. 1999) in peritoneal leukocytes from adult mice.

With respect to the adherence and chemotaxis of lymphocytes, two functions that these cells share with phagocytes, the changes with ageing were similar to those in macrophages. In previous studies we have also observed an age-related increase of adherence and decrease of chemotaxis in lymphocytes from old mice, adult prematurely ageing mice and old men and women (Guayerbas et al. 2002b, Viveros et al. 2007, Arranz et al. 2008, De la Fuente et al. 2008, De la Fuente and Miquel 2009). Similar changes have been shown in peritoneal lymphocytes from mice with endotoxic shock, a model of acute oxidative stress situation (De la Fuente and Victor 2000). The presence of NAC *in vitro*, decreased the adherence, at least with 0.1 to 2.5 mM and at 10 min of incubation, but it did not modify the chemotaxis in lymphocytes from old mice. It is interesting to consider that in peritoneal lymphocytes from adult mice, at 1 mM of NAC *in vitro* increases the adherence capacity of these cells, as well as their chemotaxis (De la Fuente et al. 2011b).

The role as immuno-modulators of antioxidants, such as NAC, bringing back altered immune function to more optimum values, has been observed in previous studies. Thus, in mice with lethal endotoxic shock, in which the peritoneal lymphocytes show increased adherence and depressed chemotaxis, NAC decreased adherence and increased chemotaxis; however, this antioxidant increased both functions in control animals (De la Fuente and Victor 2000). Moreover, in lymphocytes from chronologically adult mice, but with premature ageing, which showed higher adherence capacity than these cells from the non prematurely ageing partners, NAC *in vitro* decreased this function in cells from the prematurely ageing but increased the function in those of non-prematurely ageing animals (Puerto et al. 2002).

In conclusion, our results support that NAC acts directly on the leucocytes and that the positive effects on the immune cell functions shown after administration of diet supplemented with NAC could be due, at least in part, to this direct effect and not only to the increase of intracellular GSH levels that produce NAC (Arranz et al. 2008). Thus,

NAC may have benefits above other antioxidants probably because of both its direct and GSH-mediated effects. Moreover, NAC increases *in vitro*, at least with concentrations of 0.5 mM, the activity of antioxidant enzymes such as catalase (Pomaki et al. 2005). This capacity of up-regulation of intracellular antioxidant defences has been proposed as a better way of improving the antioxidant status of the organism than the supplementation with higher amount of antioxidants (Viña et al. 2007). Thus, the NAC administration could be proposed as a good strategy to slowdown ageing and therefore, reach a healthy longevity. However, more studies are required to clarify if the administration of antioxidants to old subjects is useful or not to control the rate of ageing. The results, although in the case of NAC are not as contradictory as with other antioxidants, are still not conclusive, and the issue of the amount of antioxidant effective (Halliwell 2009) need to be investigated further.

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REFERENCES

Alonso-Fernandez P, De la Fuente M. Role of the immune sytem in aging and longevity. Curr Aging Sci. 4:78-100, 2011.

Anderson R. Effects of ascorbate on leukocytes: Part II. Effects of ascorbic acid and calcium and sodium ascprbate on neutrophil phagocytosis and post-phagocytic metabolic activity. S Afr Med J. 56: 401-404, 1979.

Arranz L, Caamano J, Lord JM, De la Fuente M. Preserved immune functions and controlled leukocyte oxidative stress in naturally long-lived mice: Possible role of nuclear factor-kappa B. J Gerontol A Biol Sci. 65A(9):941-950, 2010.

Arranz L, Fernandez C, Rodriguez A, Ribera JM, De la Fuente M. The glutathione precursor N-acetylcysteine improves immune function in postmenopausal women. Free Radic Biol Med. 45: 1252-1262, 2008.

Atalay M, Marnila P, Lilius EM, Hanninen O, Sen CK. Glutathione-dependent modulation of exhausting exercise-induced changes in neutrophil function of rats. Eur J Appl Physiol. 74:342-347, 1996.

Aw D, Silva AB, Palmer DB. Immunosenescence: emerging challenges for an ageing population. Immunology 120: 435-46, 2007.

Bendich A., Interaction between antioxidant vitamin C and E and their effect on immune responses. In Miquel J, Quintanilla AT, Weber h, (eds), Handbook of free radicals and antioxidants in biomedicine. II. Boca Raton CRC press FL, pp153-160, 1989.

Blanco B, Ferrández MD, Correa R, Del Rio M, Guaza C, Hernanz A, De la Fuente M. Changes in several functions of murine peritoneal macrophages by N-acetylcysteine and thioproline ingestion. Comparative effect between two strains of mice. BioFactors 10:179-185, 1999.

Boyden SV. The chemotaxis effect of mixtures of antibody and antigen on polymorphonuclear leukocytes. J Exp Med. 115: 453-456, 1962.

De Flora S, Izzoti A, D'Agostini F, Cesarone CF. Antioxidant activity and other mechanisms of thiol involved in chemoprevention of mutation and cancer. Am J Med. 91: 122-130, 1991.

De la Fuente M. Changes in the macrophage function with aging. Comp Biochem Physiol. 81 A: 935-938, 1985.

De la Fuente M. Effects of antioxidants on immune system ageing. Eur J Clin Nutr. 56: S5-S8, 2002.

De la Fuente M. Role of neuroimmunomodulation in aging. Neuroimmunomodulation 15: 213-23, 2008.

De la Fuente M. Murine models of premature ageing for the study of diet-induced immune changes. Improvement of leukocyte functions in two strains of old prematurely ageing mice by dietary supplementation with sulphur-containing antioxidants. Proc Nutr Soc. 69: 651-659, 2010.

De la Fuente M, Baeza I, Guayerbas N, Puerto M, Castillo C, Salazar V, Ariznavarreta C, F-Tresguerres JA. Changes with aging in several leukocyte functions of male and female rats. Biogerontol. 5: 389-400, 2004a.

De la Fuente M, Carazo M, Correa R, Del Rio M. Changes in macrophage and lymphocyte functions in guinea-pigs after different amounts of vitamin E ingestion. Brit J Nutr. 84:25-29, 2000.

De la Fuente M, Guayerbas N, Catalán MP, Victor VM, Miquel J. The amount of thiolic antioxidant ingestion needed to improve the immune functions is higher in aged than in adult mice. Free Radic Res. 36: 119-126, 2002.

De la Fuente M, Cruces J, Hernandez O, Ortega E. Strategies to improve the functions and redox state of the immune system in aged subjects. Curr Pharm Disease. 17: In press. 2011a.

De la Fuente M**,** Hernanz A, Guayerbas N, Puerto M, Alvarez P, Alvarado C. Changes with age in peritoneal macrophage functions. Implication of leukocytes in the oxidative stress of senescence. Cell Mol Biol. 50: OL683-OL690, 2004b.

De la Fuente M, Hernanz A, Guayerbas N, Victor VM, Arnalich F. Vitamin E ingestion improves several immune functions in elderly men and women. Free Radic Res. 42: 272-280, 2008.

De la Fuente M, Hernanz A, Vallejo MC. The immune system in the oxidation stress conditions of aging and hypertension. Favorable effects of antioxidants and physical exercise. Antioxidants &Redox Signals 7: 1356-1366, 2005.

De la Fuente M Hernanz A, Viniegra S, Miquel J. Sulfur-containing antioxidants increase in vitro several functions of lymphocytes from mice. Int Immunopharmacol. 11 (6): 661-669. 2011b.

De la Fuente M, Miquel J. An update of the oxidation-inflammation theory of aging. The involvement of the immune system in oxi-inflamm-aging. Curr Pharm Design. 15: 3003-3026, 2009.

De la Fuente M, Victor VM. Antioxidants as modulators of immune function. Immunol Cell Biol. 78: 49-54, 2000.

Del Rio M, Ruedas G, Medina S, Victor VM, De la Fuente M. Improvement by several antioxidants of macrophage function in vitro. Life Sciences 63: 871-881, 1998.

Dodd S, Dean O, Copolov DL, Malhi GS, Berk M. N-acetylcysteine for antioxidant therapy: pharmacology and clinical utility. Expert Opin Biol Ther. 8:1955-1962, 2008.

Dröge W. Aging-related changes in the thiol/disulfide redox state: implications for the use of thiol antioxidants. Exp Gerontol. 37: 1333-1345, 2002.

Dröge W. Oxidative stress and ageing: is ageing a cysteine deficiency syndrome? Phil Trans R Soc B. 360: 2355-2372, 2005.

Dröge W, Breikreuz R. Glutathione and immune function. Proc Nutr Soc. 59: 595-600, 2000.

Gressier B, Cabanis A, Lebegue S, Brunet C, Dine T, Luyckx M, Cazin M, Cazin JC. Decrease of hypochlorous acid and hydroxyl radical generated by stimulated human neutrophils: comparisons in vitro of some thiol-containing drugs. Methods Find Exp Clin Pharmacol. 16: 9-13, 1994.

Guayerbas N, Catalán M, Victor VM, Miquel M, De la Fuente M. Relation of behaviour and macrophage function to life span in a murine model of premature immunosenescence Brain Behav Res. 134: 41-48, 2002a.

Guayerbas N, Puerto M, Alvarado C, De la Fuente M. Effect of diet supplementation with N-acetylcysteine on leukocyte functions in prematurely ageing mice. J Appl Biomed. 3: 199-205, 2005.

Guayerbas N, Puerto M, Alvarez P, De la Fuente M. Improvement of the macrophage functions in premature ageing mice by a diet supplemented with thiolic antioxidants. Cell Mol Biol. 50: OL677-OL681, 2004.

Guayerbas N, Puerto M, Ferrandez MD, De la Fuente M. A diet supplemented with thiolic antioxidants improves leukocyte function in two strains of prematurely ageing mice. Clin Exp Pharmacol Physiol, 29: 1009-1014, 2002b.

Halliwell B. The wandering of a free radical. Free Radic Biol Med. 46: 531-542,2009.

Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol. 2: 298-300,1956.

Hernanz A, Collazos ME, De la Fuente M. Effect of age, culture media and lymphocyte presence in ascorbate content of peritoneal macrophages from mice and guinea pigs during phagocytosis. Int Arch Allergy appl Immunol. 91: 166-170,1990.

Hernanz A, Fernández-Vivancos E, Montiel C, Vázquez JJ, Arnalich F. Changes in the intracellular homocysteine and glutathione content associated with aging. Life Sci. 67: 1317-1324, 2000.

High KP. Infection as a cause of age-related morbidity and mortality. Ageing Res Rev. 3: 1-14, 2004.

Hirokawa J, Sakaue S, Furuya Y, Ishii J, Hasegawa A, Tagami S, Kawakami Y, Sakai M, Nishi S, Nishihira J. Tumor necrosis factor-alpha regulates the gene expression of macrophage migration inhibitory factor through tyrosine kinase-dependent pathway in 3T3-L1 adipocytes. J Biochem. 123:733-739, 1998.

Jariwalla RJ, Harakeh S, Ascorbic acid: Biochemistry and Biomedical Cell Biology, in Harris JR (ed) Plenum press New York; pp: 215-231, 1996.

Karlsson H, Nava S, Remberger M, Hassan M, Ringden O. Eur J Immunol. 41: 1143-53, 2011.

Knight JA. Review: Free Radicals, Antioxidants, and Immune System. Ann Clin Lab Sci. 30: 145-58, 2000.

Lavie L. Sleep-disordered breathing and cerebrovascular disease:a mechanistic approach. Neurol Clin. 23: 1059-75, 2005.

Millea PJ. N-acetylcysteine: multiple clinical applications. Am Fam Physician. 80: 265-269, 2009.

Miquel J. An update on the oxygen stress-mitochondrial mutation theory of aging: genetic and evolutionary implications. Exp Gerontol. 33: 113-26, 1998.

Miquel J, Economos AC, Fleming J, Johnson JEJr. Mitochondrial role in cell aging. Exp Gerontol. 15:575-91, 1980.

Pomaki M, Mota MJ, De la Fuente M, Berger J, Effect of thiolic antioxidants on in vitro mouse peritoneal macrophage functions. Comp Clin Path. 13: 176-181, 2005.

Puerto M, Guayerbas N, Victor VM, De la Fuente M. Effects of N-acetylcysteine on macrophage and lymphocyte functions in a mouse model of premature ageing. Pharmacol Biochem Behavior 73: 797-804, 2002.

Victor VM, De la Fuente M. N-acetylcysteine improves in vitro the function of macrophages from mice with endotoxin-induced oxidative stress. Free Radic Res. 36: 33-45, 2002.

Victor VM, De la Fuente M. Immune cells redox state from mice with endotoxin-induced oxidative stress. Involvement of NF-kB. Free Radic Res.37: 19-27, 2003.

Victor VM, Rocha M, Esplugues JV, De la Fuente M. Role of free radicals in sepsis: Antioxidant therapy. Curr Pharm Des. 11: 3141-3158, 2005.

Viña J, Gomez-Cabrera MC, Borras C. Fostering antioxidant defences: up-regulation of antioxidant genes or antioxidant supplementation?. British J Nutr. 98:S36-S40, 2007.

Viveros MP, Arranz L, Hernanz A, Miquel J, De la Fuente M. A model of premature ageing in mice based on altered stress-related behavioural response and immunosenescence. Neuroimmunomodulation 14: 157-162, 2007.

Wayne SL, Rhyne RL, Garry PJ, Goodwin JS. Cell-mediated immunity as a predictor of morbidity and mortality in subjects over 60. J Gerontol. 45: M45-48, 1990.

Winterbourn CC. Superoxide as an intracellular radical sink. Free Radic Biol Med. 14:85-90, 1993.

Yoon SO, Yun ChH, Cheng AS. Dose effect of oxidative stress on signal transduction in aging. Mech Ageing Develop. 50: 1-8, 2002.

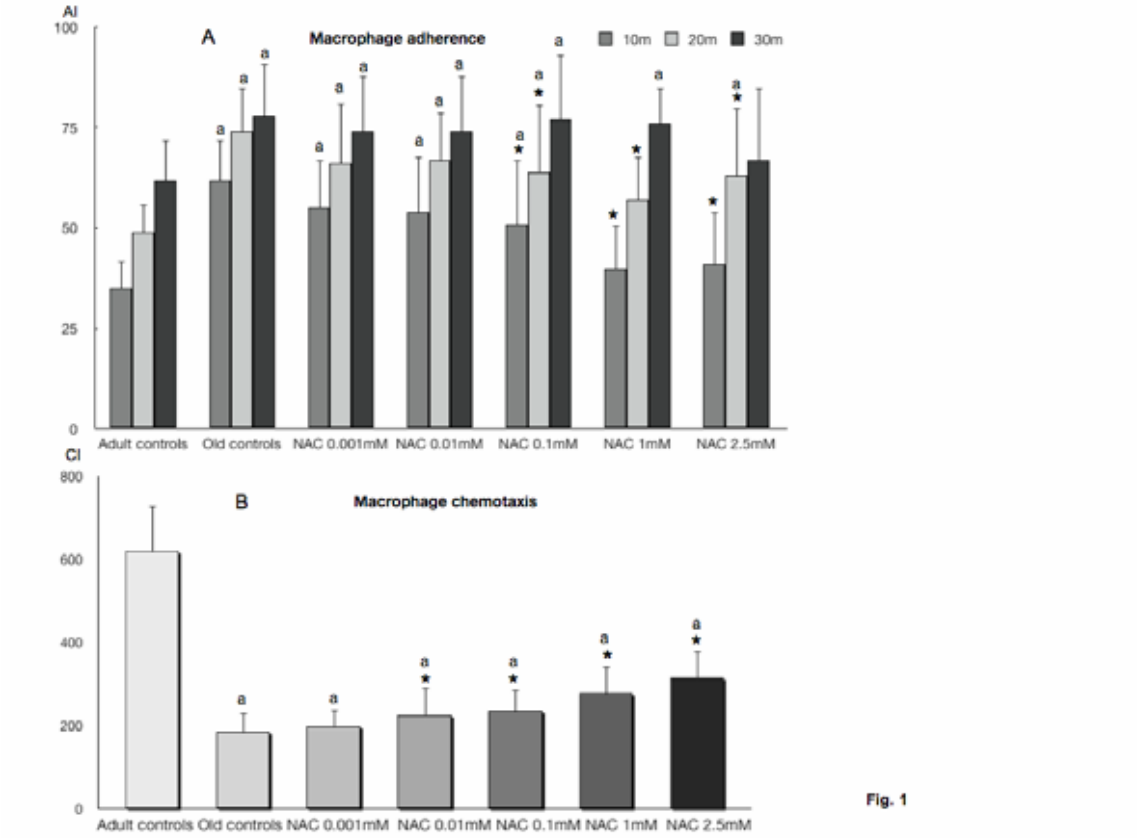


Fig. 1. Adherence of macrophages (adherence index: AI) at 10, 20 and 30 min of incubation (A) and Chemotaxis of macrophages (chemotaxis index: CI) (B) in peritoneal leukocytes from old BALB/c mice incubated with 0.001, 0.01, 0.1,1 and 2.5mM of N-acetylcysteine (NAC), as well as those indexes in cells from adult mice. Each column represents the mean ± SD of 12 values corresponding to 12 animals, with each value being the mean of duplicate assays. *Statistically significant as compared to the corresponding old control values, and [a] statistically significant as compared to the corresponding values in adult controls.

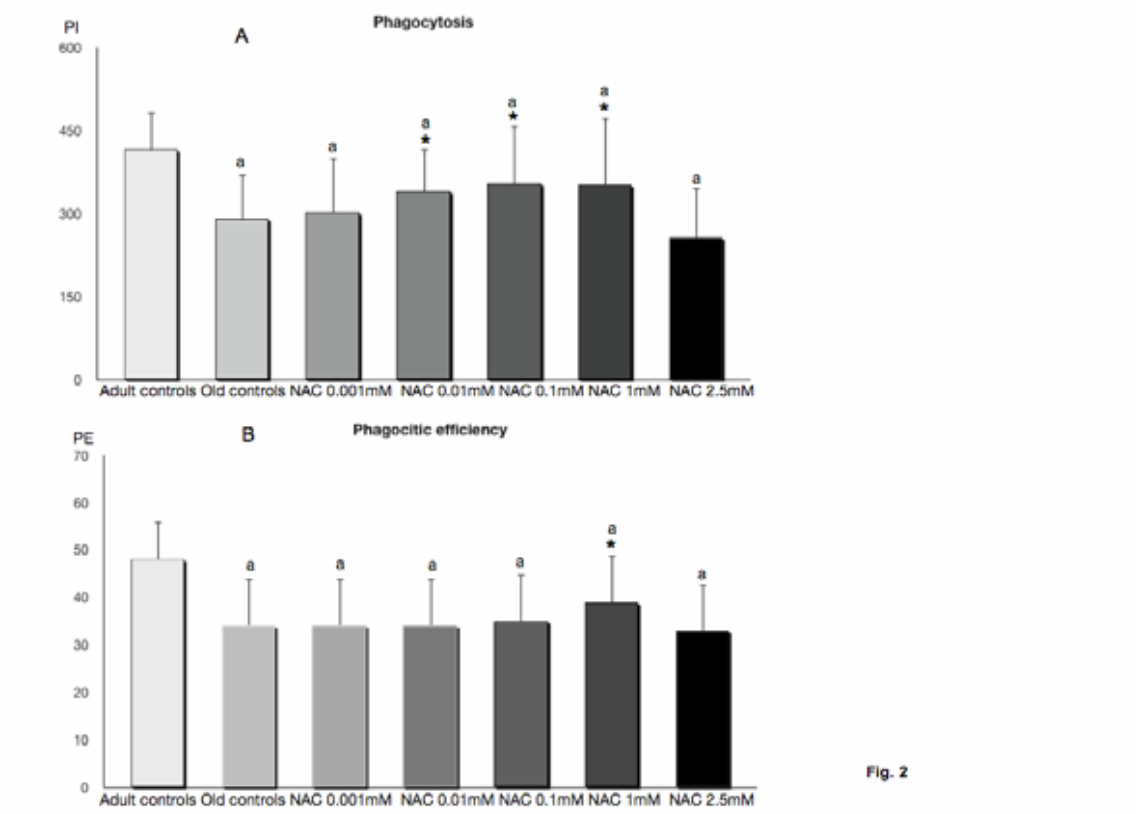
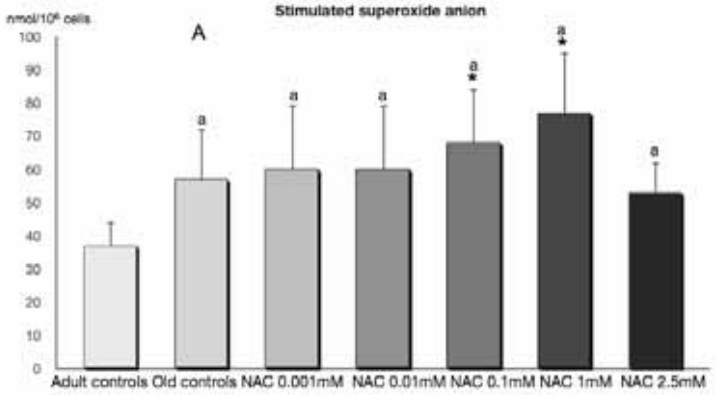


Fig. 2

Fig. 2. Phagocytosis of latex particles (Phagocytosis Index (PI): number of particles ingested by 100 macrophages) (A), and Phagocytic efficiency (Phagocytic Efficiency Index (PE): number of macrophages ingesting at least one particle per 100 macrophages) (B) in peritoneal leukocytes from old BALB/c mice incubated with 0.001, 0.01, 0.1,1 and 2.5 mM of N-acetylcysteine (NAC), as well as those indexes in cells from adult mice. Each column represents the mean ± SD of 12 values corresponding to 12 animals, with each value being the mean of duplicate assays. *Statistically significant as compared to the corresponding old control values, and [a] statistically significant as compared to the corresponding values in adult controls.



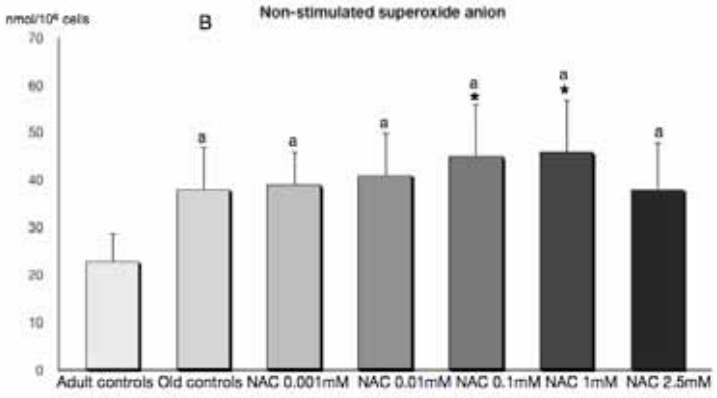


Fig. 3

Fig. 3. Superoxide anion levels (nmol/10 6 cells) of samples stimulated with latex particles (Stimulated superoxide anion) (A) and without latex particles (Non-stimulated superoxide anion) (B) in peritoneal leukocytes from old BALB/c mice incubated with 0.001, 0.01, 0.1,1 and 2.5 mM of N-acetylcysteine (NAC), as well as those indexes in cells from adult mice. Each column represents the mean ± SD of 12 values corresponding to 12 animals, with each value being the mean of duplicate assays. *Statistically significant as compared to the corresponding old control values, and [a] statistically significant as compared to the corresponding values in adult controls.

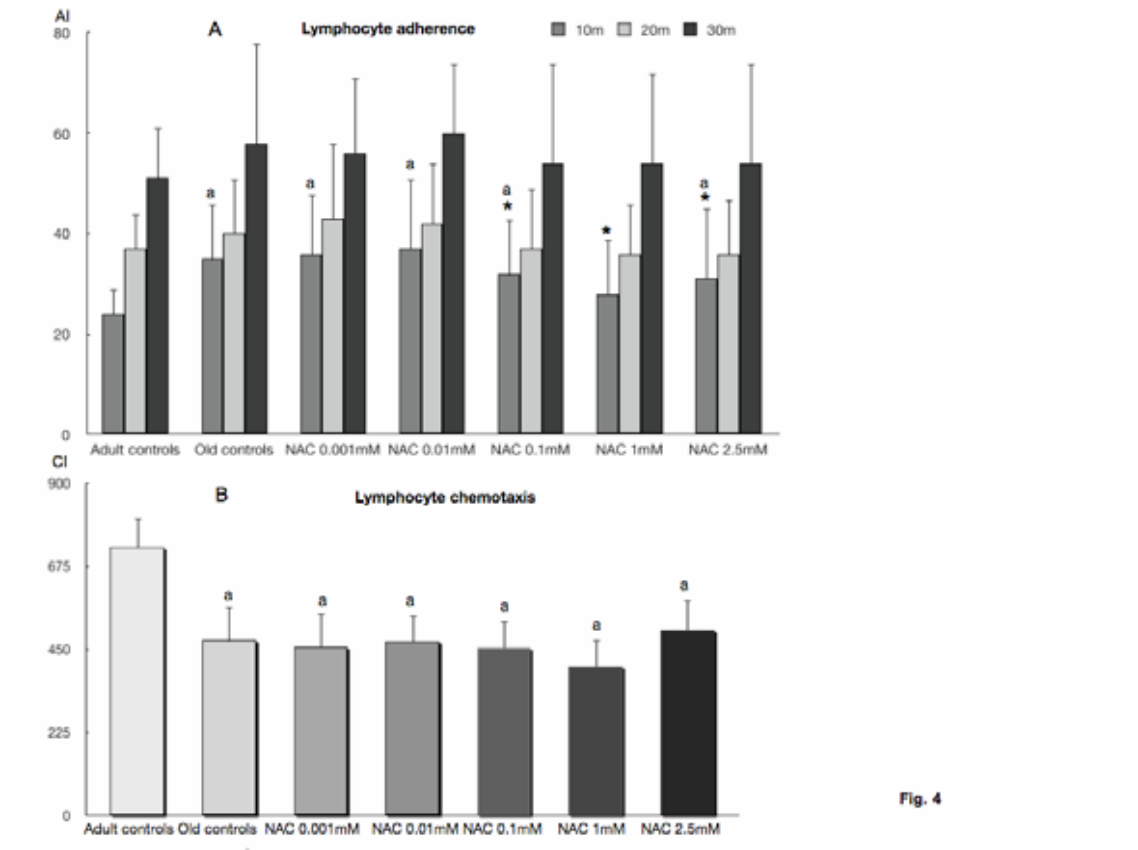


Fig. 4. Adherence of lymphocytes (adherence index: AI) at 10, 20 and 30 min of incubation (A) and Chemotaxis of lymphocytes (chemotaxis indexes: CI) (B) in peritoneal leukocytes from old BALB/c mice incubated with 0.001, 0.01, 0.1,1 and 2.5 mM of N-acetylcysteine (NAC), as well as those indexes in cells from adult mice. Each column represents the mean ± SD of 12 values corresponding to 12 animals, with each value being the mean of duplicate assays. *Statistically significant as compared to the corresponding old control values, and [a] statistically significant as compared to the corresponding values in adult controls.