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Vitamin C and vitamin C plus E improve the immune function in the elderly

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ABSTRACT

With aging the immune response is impaired. This immunosenescence, in which an alteration of the redox state of the immune cells appears, is involved in the rate of aging. Since leukocyte function is a good marker of health and predictor of longevity, the effects of daily oral administration of the antioxidant vitamin C (500 mg), or both vitamin C (500 mg) and vitamin E (200 mg) on several blood neutrophil (adherence, chemotaxis, phagocytosis, and superoxide anion levels) and lymphocyte (adherence, chemotaxis, proliferation, interleukin-2 secretion and natural killer activity) functions were studied in healthy elderly men and women. These parameters were analysed before supplementation, after 3 months of supplementation, and 6 months after the end of supplementation. The results showed that vitamin C, in elderly participants, improved the immune functions studied which achieved values close to those of young adults. These effects were maintained in several functions after 6 months without supplementation. Similar effects were found in the elderly supplemented with both vitamin C and E. Thus, a short period of vitamin C or vitamin C and E ingestion, with the doses used, improves the immune function in elderly men and women and could contribute to a healthy longevity.

1. Introduction

Aging is a progressive and general impairment of the physiological systems including the immune system. It is presently accepted, that almost every component of the immune system undergoes striking age-associated re-structuring, leading to changes that may include diminished as well as enhanced functions. The final result of this denominated immunosenescence, is an age-related deterioration of the defensive system, which explains the increase of cancer and the susceptibility and vulnerability to infections among aged subjects, these being their most common causes of illness and death (High, 2004; De la Fuente and Miquel, 2009; Desai et al., 2010; De la Fuente et al., 2011; Bauer and De la Fuente, 2016). With age there is a pronounced decrease of adaptive immunity carried out by lymphocytes, the functions of which, especially in T-cells, are impaired. With respect to innate immunity, natural killer (NK) cells show a decreased cytotoxicity, and phagocytes display a decline of several of their activities such as phagocytosis and chemotaxis (De la Fuente and Miquel, 2009; Desai et al., 2010; De la Fuente et al., 2011; Bauer and De la Fuente, 2016; Martinez de Toda et al., 2016; Oh et al., 2019).

In addition, it has been shown that the competence of the immune system is an excellent marker of health (Wayne et al., 1990) and several age-related changes in immune functions have been linked to longevity. Thus, individuals who live longer in good health, such as centenarians or extremely long-lived animals, show values for several immune functions similar to those in the corresponding healthy adults. These and other results have allowed us to propose those functions as markers of the rate of aging of each individual, that is to say, they are useful for the determination of "biological age" (De la Fuente and Miquel, 2009; Martinez de Toda et al., 2016).

Among all the many theories about how the aging process occurs, the free radical-oxidation theory is one of the most widely accepted (Harman, 1956; De la Fuente and Miquel, 2009; Oliveira et al., 2010; Martinez de Toda et al., 2020). More recently, the theory of oxidation-inflammation has been proposed (De la Fuente and Miquel, 2009). Thus, aging is the result of damage accumulation by deleterious oxidation in biomolecules, as a consequence of the age-related chronic oxidative stress (imbalance between oxidant generation and antioxidant defences, in favor of the later). Since oxidation and inflammation are two related processes, an inflammatory stress also appears with age (De la Fuente, 2018). This oxidative-inflammatory stress affects all cells of the organism but especially those of the homeostatic

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systems, such as the immune system. Moreover, this theory of oxidation-inflammation suggests that, as the immune cells are an important source of oxidant and inflammatory compounds, which are used to carry out their defensive function, if this production is not well regulated, it can affect the oxidative and inflammatory state of the organism and, consequently, its rate of aging (De la Fuente and Miquel, 2009).

In each cell an adequate oxidant-antioxidant balance is very important for maintaining its function, and more so in the immune cells, in which the reactive oxygen species (ROS) play a pivotal role not only in regulating many processes, but also in destroying pathogens and tumoral cells. Thus, the antioxidants of these cells are used when they carry out their defensive action. Because of this, it is not surprising that an antioxidant deficit has been related to impaired immune responses, leading to frequent and severe infections that result in increased mortality (De la Fuente and Miquel, 2009; De la Fuente et al., 2011; Paiva and Bozza, 2014; Amir-Aslani and Ghobadi, 2016). An important number of studies show that the ingestion of diets with adequate levels of antioxidants such as vitamins E and C, β-carotene, polyphenols and others, are able to retard or prevent the oxidative damage (Calabrese et al., 2010; De la Fuente et al., 2011) and therefore the general physiological impairment associated with aging, and in particular immunosenescence. This suggests that these diets are a good way to improve the immune system in elderly subjects (De la Fuente and Miquel, 2009; De la Fuente et al., 2011; Pae and Wu, 2017).

Ascorbic acid (vitamin C) is a hydro-soluble antioxidant present in the extracellular fluid and the cytosolic compartment of the cell, which carries out a variety of functions especially in immune homeostasis. It is highly concentrated in leukocytes and declines during infections and stress, since it is rapidly used in their defensive work. Decreased concentrations of this vitamin in the immune cells are associated with a lower functional capacity of these cells. In addition, vitamin C has been shown to stimulate, in vitro and after supplementation trials, several immune cell functions, contributing to the maintenance of the redox integrity of leukocytes during the inflammatory response (Hernanz et al., 1990; Wintergerst et al., 2006; Stadler et al., 2007; De la Fuente and Miquel, 2009; De la Fuente et al., 2011; Carr and Maggini, 2017; Ang et al., 2018; Spoelstra-de Man et al., 2018; Van Gorkom et al., 2018; Bozonet and Carr, 2019).

Vitamin E is the most important lipid-soluble antioxidant present in the biological membrane and the first line of defence against lipid peroxidation. Several in vitro and in vivo studies in animals and human models under normal and disease conditions have shown the immunomodulatory effects of vitamin E improving, in general, the function of immune cells (De la Fuente and Miquel, 2009; De la Fuente et al., 2011; Galli and Azzi, 2010; Lee and Han, 2018).

Although the effect of antioxidant supplementation on the immune functions in the elderly is a subject of great interest, and it is accepted that micronutrients such as vitamin E and C provide additional benefits to immunocompromised persons, there has been little relevant research, especially on healthy men and women (De la Fuente et al., 2008; Meydani et al., 2018; Elmadfa and Meyer, 2019). It is known that older people show the highest risk of both poor nutrition (with an insufficient intake of these micronutrients) and increased oxidative stress (De la Fuente and Miquel, 2009; De la Fuente et al., 2011; Corcoran et al., 2019; Martinez de Toda et al., 2020). Thus, it has been observed that low blood concentrations of vitamin C in old population are a strong predictor of mortality (Spoelstra-de Man et al., 2018). Although previous studies have shown clearly that vitamin E supplementation improves several immune functions in the elderly (De la Fuente et al., 2008; Meydani et al., 2018), the effects of vitamin C in this context have not been investigated.

In addition, several studies show the relevance of the intake of supplementation with more than one antioxidant (Alvarado et al., 2006; Shymanskyy et al., 2016; Gombart et al., 2020). However, the effect of a combination of vitamin C and vitamin E has scarcely been studied. Preliminary research on elderly women with a vitamin C and vitamin E supplementation showed an improvement of several immune functions (De la Fuente et al., 1998). In addition, in a neurodegenerative illness such as Alzheimer's disease (AD), the combination of vitamins C and E has been associated with a decrease in the prevalence and incidence of the disease, that was not evidenced by the use of vitamin C or E supplements alone (Zandi et al., 2004).

In view of the above, we conducted a study to determine the effect of vitamin C supplementation (500 mg/day) and vitamin C (500 mg/day) plus vitamin E (200 mg/day) for a short period of time (3 months), on several functions of immune cells in healthy elderly men and women, as well as the prevalence of these effects. Moreover, we used in parallel, adult men and women to discover if the antioxidant supplementation could improve the immune function to levels similar to those normally present at this age.

2. Materials and methods

2.1. Subjects

Groups of 44 elderly (24 women and 20 men) (mean age ± SD: 74 ± 4 years old) and 30 adult (15 women and 15 men) (mean age ± SD: 35 ± 5 years old) volunteers were used for this study. Sample size was calculated according to standard deviations from the mean of parameters in the groups under study, error of $\alpha = 0.05$, power of 80% and risk of retirements of 10%. Although the study began with 90 people, who agreed to participate in the investigation, it was completed by only 44 subjects, since many failed to continue taking the supplements or changed their life habits drastically and had to be discarded from the results. All individuals studied in the present work were Spanish and recruited from the population of Madrid. The inclusion criterion was that they were to be in a healthy condition, which was defined as absence of pathology or findings of clinical significance in general laboratory parameters. Exclusion criteria (only one required for exclusion) were severe general pathology, autoimmune diseases, cancer, anaemia, severe allergies, chemotherapy, dementia or cognitive alteration, chronic respiratory disease, hypertension, diabetes, consumption of excess of alcohol or of drugs, life expectancy inferior to one year, poor collaboration level, and intake of vitamins, antioxidants or any drug influencing the immune system as well as high fruit/juice and vegetable consumption (> 5 servings/day). All elderly subjects were selected according to the “SENIEUR” protocol (Lighthart et al., 1984; Zandi et al., 2004). The participating men and women were not hospitalized during the course of the investigation, and they carried on an active life. They resided in their homes and consumed a Spanish standard diet, very close to the Mediterranean diet. There was no change in the diet throughout the study for any of the subjects. The adult control group (30 volunteers) was constituted by relatives, friends or colleagues of the research group. The inclusion criterion was to be 35 ± 5 years old in healthy condition. Exclusion criteria were the same as those for the experimental groups but included pregnancy, intake of estrogens, or performance of endurance training shortly before admission.

All participants received information about the purpose of the study and gave written consent for their blood samples to be used for scientific research. Informed consent was sought from potential participants before the beginning of any specific procedure relative to the study. Interviews were conducted in a private room of the Department of Internal Medicine of the La Paz Hospital by the Dr. Francisco Arnalich. This study was approved by the Ethics Committee of the La Paz Hospital of Madrid and was in agreement with the principles of the Declaration of Helsinki approved by the World Medical Association and the official Spanish (Low 14/2007 and RD 1716/2011) regulations.

2.2. Clinical interviews

A total of 3 interviews were performed with the subjects belonging to the experimental groups. The first interview was done on day 1 for

the final selection of participants, whereas the following were carried out to check the correct development of the study. In the first interview the volunteers were randomly assigned to the vitamin C group or to the vitamin C plus vitamin E group. The second visit was done 3 months after the beginning of antioxidant intake (the last day of the treatment), the third was carried out 6 months after the end of the treatment. Blood samples were drawn during each clinical interview.

2.3. Collection of blood samples

Peripheral blood samples were always collected at the same time, from 9 to 10 a.m., in order to control the effects of circadian variations on immune parameters, during the course of each Clinical Interview, in tubes with EDTA (BD Vacutainer Systems, Spain). Blood samples of subjects from the experimental groups were taken before (BS), after 3 months of supplementation (S) and 6 months after the end of supplementation, without intake of vitamin C or vitamin C and E (post-supplementation, PS). Samples from adult controls were drawn once only, spread throughout the whole study. At each time point 5 men and 5 women, all healthy adults, were studied and 15 men and 15 women were used as controls. The experiments were carried out without knowing if the samples came from the control or the supplemented population.

2.4. Vitamin C, and vitamin C and E supplementations

A group of 22 elderly (12 women and 10 men) received a daily supplement of 500 mg of vitamin C (Bayer) and another group of 22 elderly (12 women and 10 men) received a daily supplement of 500 mg of the vitamin C and 200 mg of dl-alpha-tocopherol (Alcala Farma) for three months. The doses were chosen based on previous studies (Weber et al., 1996; Meydani et al., 2004; Wintergerst et al., 2006; De la Fuente et al., 2008). Since intakes of up to 1.000 mg/day of vitamin C show a favourable effect on immune response (Wintergerst et al., 2006), and it has been indicated that an intake of 200 mg/day at least is needed to increase immune functions (Weber et al., 1996), a dose of 500 mg/day of vitamin C was chosen for the present study. In the case of vitamin E, with controversial results on immunity (positive or without effect depending on the doses), the previously used dose of this vitamin with a good effect on the immune functions studied in the present work (De la Fuente et al., 2008), as well as with protective effects on upper respiratory infections (Meydani et al., 2004), was chosen.

2.5. Separation of blood neutrophils and lymphocytes

Peripheral blood neutrophils and lymphocytes were obtained following a method previously described (De la Fuente et al., 2008), by gradient sedimentation using 1.119 density Hystopaque (Sigma) for neutrophil separation and 1.077 density Hystopaque for lymphocytes. The cells were harvested, washed twice in Hank's medium for neutrophils or RPMI medium (Gibco, Burlington, Ontario, Canada) for mononuclear cells (principally lymphocytes), counted and adjusted to 5×10^5 neutrophils/ml medium and 1×10^6 lymphocytes/ml medium. Cell viability was checked by the trypan blue (Sigma) exclusion test before and after each assay and was equal or higher than 99% in all cases.

2.6. Assays of neutrophil functions

All the assays were carried out following methods previously described (De la Fuente et al., 2008). The adherence capacity of neutrophils was measured following a method, which mimics in vitro (using nylon fibre columns) the adherence of neutrophils in vivo to the vascular endothelium. Briefly, 1 mL of whole blood (diluted 1:1 with Hank's medium) was placed in a Pasteur pipette in which 50 mg of nylon fibre was packed to a height of 1.25 cm. After 10 min, the effluent had drained by gravity. The results were expressed as adherence index (A.I), which was calculated as follows:

$$A.I. = 100 - \frac{\text{neutrophils or lymphocytes per mL of effluent samples}}{\text{neutrophils or lymphocytes per mL of original samples}} \times 100$$

The chemotaxis was evaluated measuring the mobility capacity of neutrophils towards an infectious focus, using Boyden chambers with 2 compartments separated by a nitrocellulose filter (3 μm pore diameter, Millipore Iberica, Madrid, Spain). Aliquots of 300 μL of the neutrophil suspension were deposited in the upper compartment of a Boyden chamber. Formyl-met-phe-leu (Sigma, St. Louis, MO, USA), a chemoattractant agent, was put in the lower compartment at 10^{-8} M to induce chemotaxis. After 3 h of incubation at 37 °C and 5% CO_2, the filter was fixed (methanol 50% and ethanol 75%) and stained (azur-eosin-methylene blue solution, GIEMSA, PANREAC). The results were expressed as the chemotactic index (C.I.), representing the total number of neutrophils counted by optical microscopy (immersion objective) on one-third of the lower face of the filters.

The phagocytosis of inert particles (latex beads) was carried out using migration inhibition factor (MIF) plates (Sterilin, Teddington, UK). Aliquots of 200 μL of neutrophil suspension were incubated on MIF plates for 30 min and the adherent monolayer was washed with PBS (phosphate buffer saline) at 37 °C, and 20 μL latex beads (1.09 μm diluted to 1% PBS, Sigma- Aldrich) were added. After 30 min of incubation, the plates were washed, fixed (methanol 50%) and stained with azur-Eosin-Methylene Blue solution and the number of particles ingested by 100 neutrophils was determined by optical microscopy (immersion objective), and it was expressed as the phagocytosis index (P.I.).

Superoxide anion, the first response in the respiratory burst, was evaluated assessing the reduction of nitroblue tetrazolium (NBT) in neutrophils. This was carried out following the method described by De la Fuente et al. (2008) slightly modified as follows. Aliquots of 250 μL neutrophil suspension (10^6 cells/mL Hank's medium) were mixed with 250 μL NBT (1 mg/mL in Hank's solution, Sigma, St. Louis, U.S.A.), and 50 μL of Hank's medium (non-stimulated samples) or 50 μL of the latex bead suspension (1%) (stimulated samples) were added to non-stimulated or stimulated samples, respectively. After a 60 min incubation, the reaction was stopped, samples were centrifuged, supernatants discarded, and intracellular reduced NBT was extracted with dioxan. Eventually, supernatant absorbances were measured at 525 nm by spectrophotometer. The results were expressed as Absorbances.

2.7. Assays of lymphocyte function

Lymphocyte adherence and chemotaxis methods were similar to the above described in neutrophils (De la Fuente et al., 2008).

The lymphoproliferation assay was performed using a standard method, previously used by us (De la Fuente et al., 2008). The suspensions of mononuclear leukocytes were adjusted to 10^6 lymphocytes/mL of RPMI (Gibco) supplemented with gentamicin (1 mg/mL, Gibco) and 10% fetal bovine serum (FBS) (Gibco), previously inactivated by heat (30 min at 56 °C). Aliquots of 200 μL were dispensed in plates of 96 wells (Costar, Cambridge, MA, USA) and 20 μL of phytohemagglutinin (PHA, Flow) at 25 μg/mL was used as mitogen. After 48 h of incubation, 0.5 μCi/well ^{3}H-thymidine (Dupont, Boston, MA) was added, followed by another 24 h of incubation. The cells were harvested in a semi-automatic harvester and thymidine uptake was measured in a beta counter (LKB, Upsala, Sweden) for 1 min. The results were expressed as ^{3}H-thymidine uptake (cpm).

The concentration of interleukin-2 (IL-2) released by lymphocytes was determined in supernatants of the above cultures of 48 h, following a method previously described by us (De la Fuente et al., 2008). IL-2 was measured using an ELISA kit (R&D Systems, Minneapolis, MN,

USA).

The natural killer (NK) activity was evaluated following an enzymatic colorimetric assay (Cytotox 96 TM Promega, Boeringher Ingelheim, Germany) based on the determination of lactate dehydrogenase (LDH) released by the cytolysis of tumour cells (target cells: human tumour K562 cells), using tetrazolium salts (De la Fuente et al., 2008). Target cells were seeded in 96-well U-bottom culture plates at 10^4 cells/well in RPMI medium without phenol red. Effector cells (lymphocytes) were added at 10^5 cells/well, the effector/target rate being, 10/1. The plates were centrifuged at 250 *g* for 5 min to facilitate cell to cell contacts and then they were incubated for 4 h. Then, they were centrifuged again and LDH enzymatic activity was measured in 50 μL/well of supernatants by addition of the enzyme substrate and absorbance recording at 490 nm. The results were expressed as the percentages of lysis of tumour cells (% lysis), which were determined with the following equation:

$$\%lysis = \frac{E - ES - TS}{M - TS} \times 100$$

where E is the mean of absorbance in the presence of effector cells, ES the mean of absorbance of effector cells incubated alone, *TS*, the mean of absorbances in target cells, and M the mean of maximum absorbance after incubating target cells with lysis solution.

2.8. Statistical study

The results are expressed as the mean ± standard deviation (SD) of the values corresponding to subjects, each value being the mean of duplicate assays (two samples from the same blood). The data were evaluated statistically by the one-way analysis of variance (ANOVA) for paired observations, used to evaluate vitamin supplementation in the aged groups, followed by the Scheffe's F post hoc procedure. The differences due to the treatment, in each experimental group, were evaluated by the Student's *t*-test for related samples. The two-way ANOVA test for unpaired observations was used for age and gender groups, followed by the Scheffe's F test. Normality of the samples was confirmed by the Kolmogorov-Smirnov test, while the homogeneity of variances was studied by the Levene test, $P < 0.05$ being the minimum level of significance. The Sidak test with a level of significance set at $P < 0.05$ was used for post hoc comparisons.

3. Results

Several sociodemographic, physiological and biochemical characteristics of the two populations studied are shown in Table 1.

The results of the blood neutrophil activities carried out in the phagocytic process (the adherence capacity, the mobility directed to the infectious focus by a chemoattractant gradient (chemotaxis), ingestion of foreign agents, and their destruction with the help of oxygen free radicals, starting with the superoxide anion) are shown in Figs. 1 and 2. Regarding the adherence capacity of neutrophils, the aged groups before supplementation (BS) showed higher values of adherence indexes (AI) than adult controls (AC), statistical differences being higher in the male groups than in the female groups. After vitamin supplementation (S), the values of AI were significantly decreased with respect to the corresponding BS values in all male and female groups, showing values similar to those of cells from the adults. After 6 months without vitamin ingestion (PS) the values of AI are similar to those in BS in women, but they remained lower than those of BS in men. Comparing the effects of vitamin C versus vitamin C plus E, the AI values in men that only took vitamin C were lower after supplementation (S) and after 6 months without vitamin ingestion (PS) than those in men with both vitamins.

The chemotaxis indexes (CI) of neutrophils of elderly women and men, before supplementation (BS), were lower than those of the adults in all the groups. After supplementation (S), these indexes increased with respect to those found in BS, reaching values similar to the corresponding adults, with the exception of the group of vitamin C in men, in which the values were still lower than in the corresponding AC. In the PS groups the CI brought the values near those of the BS.

The phagocytosis indexes (PI), lower in neutrophils from elderly women and men than in those from the corresponding adults, increased after supplementation with vitamins in all the groups, the values being similar to those in adults or even higher as occurs in the vitamin C group of women. In the PS groups the values decreased in women with respect to those after supplementation (S), being close to the values of the BS. However, in men the values of PS were similar to those in S.

The results corresponding to the levels of superoxide anion in non-stimulated and stimulated neutrophils are shown in Fig. 2. The values in the aged BS groups were significantly higher in all the groups with respect to those in the corresponding adults. After supplementation (S) there were significant decreases in all groups. Thus, the values were similar to those in adults or even lower (this is the case in women in both groups of supplementation in non-stimulated neutrophils and in the vitamin C + E group in stimulated cells, together with men in the vitamin C + E group both in non-stimulated and stimulated neutrophils). In the PS groups the values were increased with respect to those after supplementation (S), but decreased with respect to those in BS, the values being similar to those in adults. Comparing the effects of vitamin C versus vitamin C plus E, the superoxide anion levels were lower after supplementation (S) with both vitamins than with vitamin C, in neutrophils of men, both stimulated and non-stimulated, and in stimulated neutrophils of women.

With respect to the lymphocyte functions studied, the results of adherence (A.I.) and chemotaxis (C.I.) are shown in Fig. 3. The values of A.I. of lymphocytes from elderly subjects before supplementation (BS) were higher than those from adults. After vitamin supplementation (S), the values of A.I. were decreased with respect to the corresponding BS values in women and men, showing similar values to those in cells from adults or even lower values, as is the case of the group of vitamin C in men. In PS the values of A.I. were similar to those found before supplementation (BS) (in the vitamin C + E group of women and in the vitamin C group of men) or they maintained values lower than BS (in the vitamin C group in women and in the vitamin C + E group in men). Comparing the effects of vitamin C versus vitamin C + E, the AI was lower in neutrophils of men after supplementation (S) with only vitamin C than with both vitamins. The chemotaxis of lymphocytes was lower in elderly men and women than in the adults. With vitamin supplementation (S) this function increased in cells from both men and women, the values being similar to those in adult controls. After 6 months without supplementation (PS) the values of CI decreased with respect to the values in S in all the groups, although preserving values higher than those before supplementation in the group of vitamin C in men.

The lymphoproliferative capacity in response to PHA, the IL-2

Table 1
Sociodemographic, anthropometric, physiological and biochemical characteristics of the participants.

Characteristics	Adult subjects	Old subjects
Number	30	44
Gender		
Man	15	20
Woman	15	24
Education	Bachelor's degree	High school-Bachelor's degree
Social class	Middle class	Middle class
Weight (kg)	70 ± 17	75 ± 20
Height (cm)	172 ± 12	167 ± 10
BMI (kg/m^2)	20 ± 4	24 ± 5
Glucose (mg/dL)	92.6 ± 5.3	135.67 ± 55.4
Triglycerides (mg/dL)	100.2 ± 20.1	104.2 ± 30.9
Cholesterol (mg/dL)	135.4 ± 19.8	143.3 ± 30.5
Blood preasure (mm Hg)	90 ± 5	100 ± 6

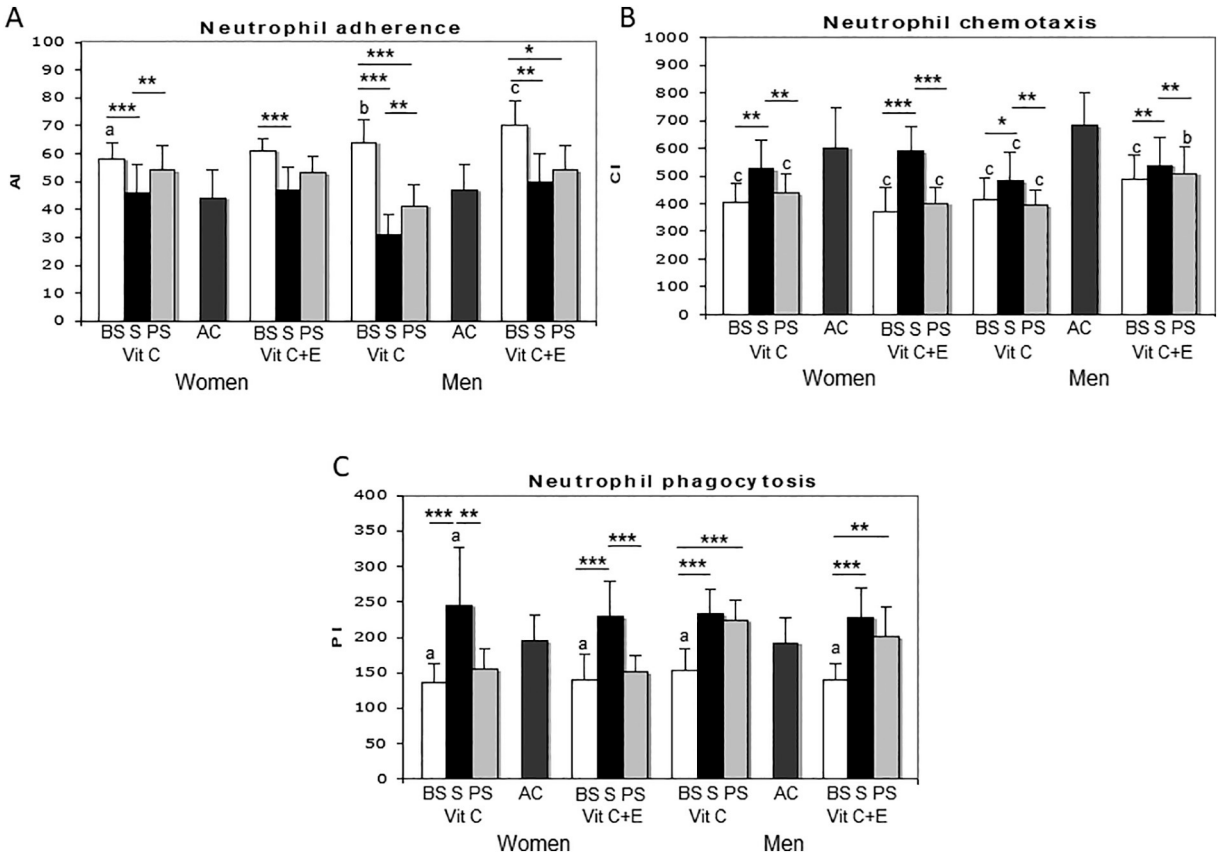

Fig. 1. Neutrophil adherence (adherence index (AI): percentage of neutrophil adherence to nylon fibre) (A), chemotaxis (chemotaxis index (CI): number of neutrophils on filter) (B) and phagocytosis (phagocytic index (PI): number of latex beads/100 neutrophils) (C) capacities of cells from adult controls (AC) and elderly subjects before (BS), after 3 months of vitamin C or vitamin C plus vitamin E supplementations (S) and 6 months after the end of supplementations without vitamin intake (PS). Each column represents the mean ± standard deviation of the values corresponding to elderly (12 women or 10 men), and adult (15 women and 15 men) subjects and each value being the mean of duplicate assays. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ with respect to the corresponding BS or S values. $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$ with respect to the corresponding AC values.

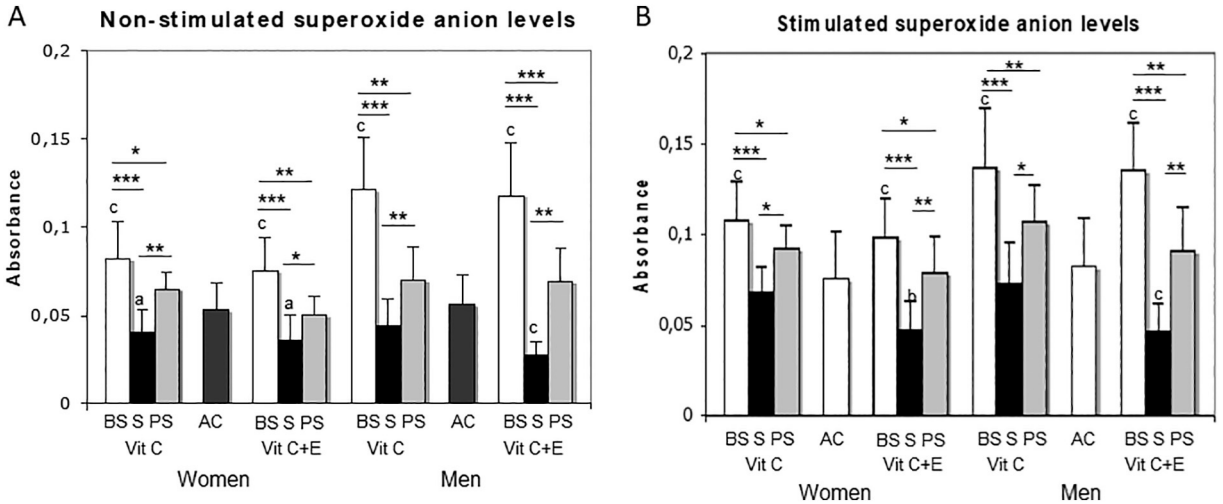

Fig. 2. Superoxide anion levels in non-stimulated and stimulated samples (with latex beads) (Absorbances) of human peripheral neutrophils from adult (controls) (AC) and elderly men and women before (BS), after 3 months of vitamin C or vitamin C plus vitamin E supplementations (S) and 6 months after the end of supplementations without vitamin intake (PS). Each column represents the mean ± standard deviation of the values corresponding to elderly (12 women or 10 men) and adult (15 women and 15 men) subjects and each value being the mean of duplicate assays. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ with respect to the corresponding BS or S values. $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$ with respect to the corresponding AC values.

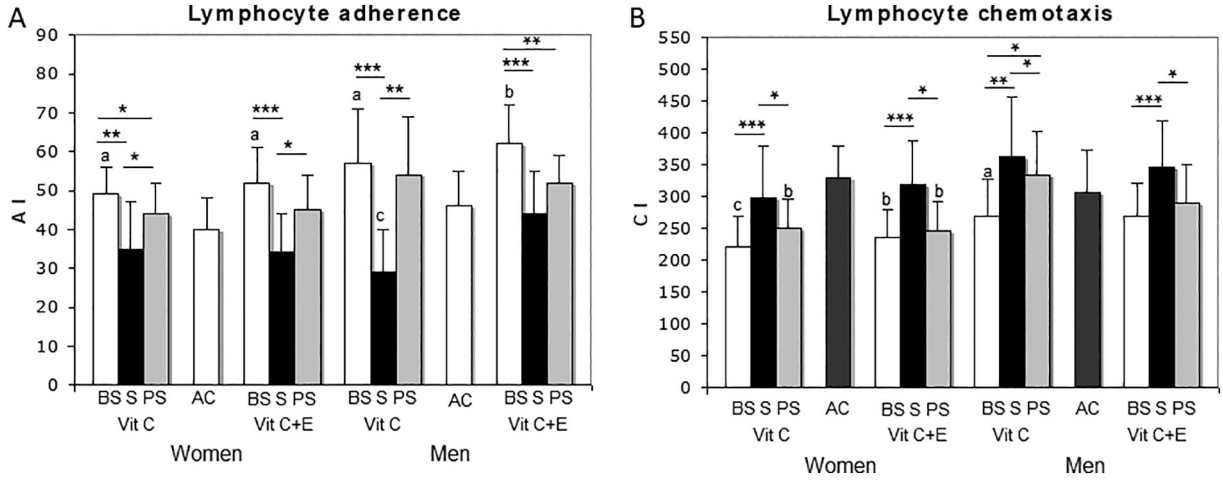

Fig. 3. Lymphocyte adherence (adherence index (AI): percentage of lymphocyte adherence to nylon fibre) (A) and chemotaxis (chemotaxis index (CI): number of lymphocytes on filter) (B) capacities of cells from adult controls (AC) and elderly subjects before (BS), after 3 months of vitamin C or vitamin C plus vitamin E supplementations (S) and 6 months after the end of supplementations without vitamin intake (PS). Each column represents the mean ± standard deviation of the values corresponding to elderly (12 women or 10 men), and adult (15 women and 15 men) subjects and each value being the mean of duplicate assays. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ with respect to the corresponding BS or S values. $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$ with respect to the corresponding AC values.

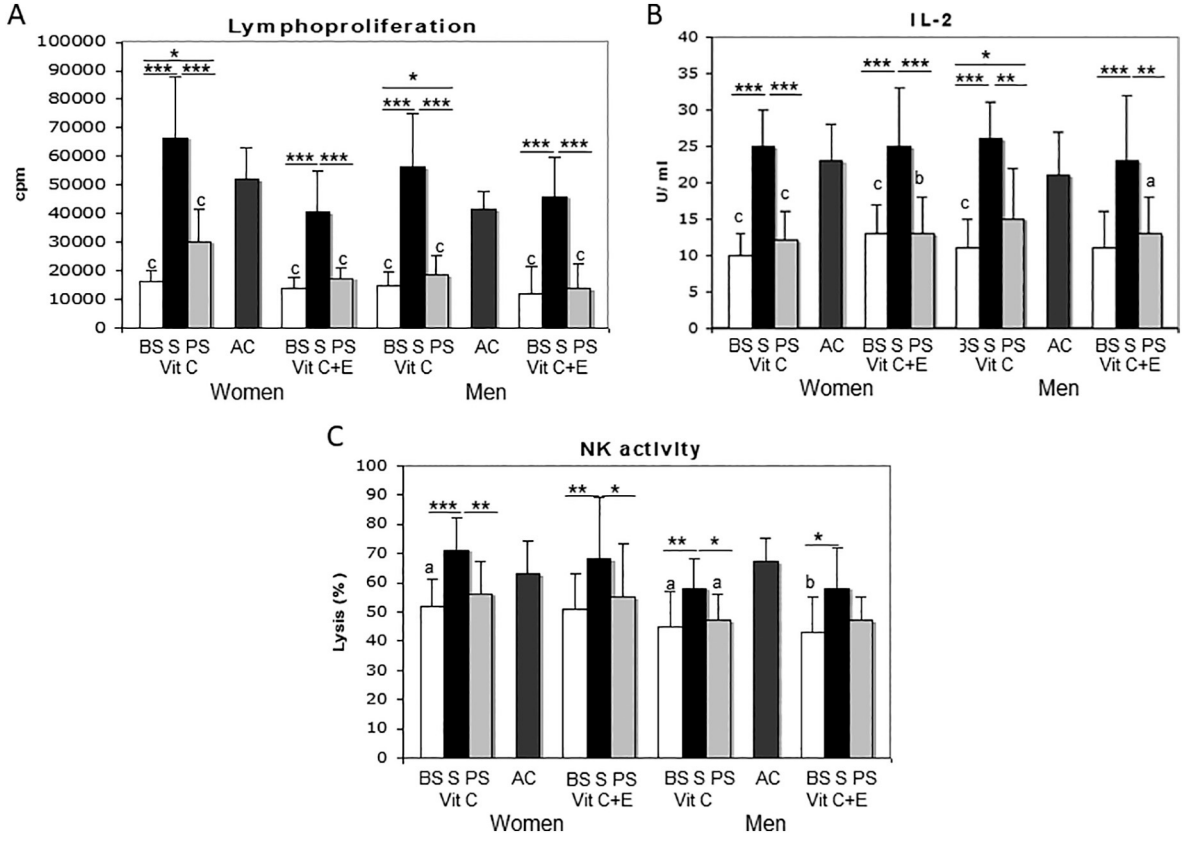

Fig. 4. Proliferation in response to PHA (counts per minute: cpm) (A), IL-2 levels (U/ml) in supernatants of PHA-stimulated cultures of lymphocytes (B) and NK activity (lysis % of human tumour cells) (C) of human peripheral lymphocytes from adult controls (AC) and elderly subjects before (BS), after 3 months of vitamin C or vitamin C plus vitamin E supplementations (S) and 6 months after the end of supplementations without vitamin intake (PS). Each column represents the mean ± standard deviation of the values corresponding to elderly (12 women or 10 men), and adult (15 women and 15 men) subjects and each value being the mean of duplicate assays. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ with respect to the corresponding BS or S values. $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$ with respect to the corresponding AC values.

release and the NK activity are shown in Fig. 4. These parameters were lower in cells from elderly men and women than in adults. After the supplementation with vitamins (S) all these functions were stimulated, showing values similar to those in adults. After 6 months without supplementation of vitamins (PS) the values decreased, being similar to those found BS in all the groups and functions with the exception of lymphoproliferation of the group of vitamin C in women and men, and in the case of IL-2 release in the vitamin C group in men. In those cases

the values were higher than in BS. Comparing the effects of vitamin C versus vitamin C plus E, the proliferation was higher after supplementation (S) and after 6 months without supplementation (PS) with vitamin C than with both vitamins, in lymphocytes of women.

4. Discussion

This work describes for the first time that a short period (3 months) of supplementation with vitamin C (500 mg/day) in elderly men and women improves several relevant functions of the immune cells in human peripheral blood, which are those that suffer an impairment with age. In addition, this is the first attempt to investigate the effects of the same short period of supplementation with vitamin C and E not only in elderly women, in which we had studied only a few immune parameters previously, and with a higher dose of vitamin C and period of supplementation than those used here (De la Fuente et al., 1998; De la Fuente and Víctor, 2000), but also in men, and adding adult control groups.

With vitamin C plus E, in general, similar results to those with only vitamin C have been obtained, although in functions such as the adherence and proliferation of lymphocytes, a more positive effect of the vitamin C supplementation alone was found. In the present work we have also investigated, if after a period of 6 months without these supplementations, the effects are maintained, which occurs in several functions. This is more frequent with vitamin C than with vitamin C plus E. Since in all the cases the values of the immune functions after intake of the vitamins were closer to those of adults, these antioxidants seem to be modulators of immune functions and not merely stimulators of them, as has been observed with these and other antioxidants (De la Fuente and Víctor, 2000; De la Fuente et al., 2008).

The values of the functions studied in elderly subjects with respect to those in adults confirm the state of immunosenescence of the healthy elderly men and women investigated before the intake of antioxidants. Thus, T cells, which are considered to be the most susceptible to immunosenescence, showed in both elderly men and women a lower proliferation response to the mitogen PHA, one of the central events implicated in the development of the immune response, as well as in its IL-2 release, than the corresponding adult cells. This in agreement with previous results (De la Fuente and Miquel, 2009; Bauer and De la Fuente, 2016; Martinez de Toda et al., 2016). As regards functions of the non-specific immune response carried out by immune cells, most previous research shows also a decrease in antitumoral NK citotoxicity, in chemotaxis and ingestion of phagocytes as well as in chemotaxis of lymphocytes (De la Fuente and Miquel, 2009; Bauer and De la Fuente, 2016; Martinez de Toda et al., 2016). However, adherence capacity, of both lymphocytes and phagocytes, increases with aging as several studies have shown (De la Fuente et al., 2008; De la Fuente and Miquel, 2009). With respect to the age-related changes in the levels of superoxide anion, although there are contradictory data, previous results have also shown an increase of this anion in neutrophils from elderly men and women (De la Fuente et al., 2008; Arranz et al., 2008; Martinez de Toda et al., 2020). All these results show that the men and women participants in the present study had the typical immunosenescence corresponding to their chronological age.

Since immunosenescence is a consequence of oxidative stress (De la Fuente and Miquel, 2009; De la Fuente, 2018), diet supplementation with antioxidants has been investigated as a way to prevent or even reverse that age-related immune dysfunction, thus increasing health and therefore life span (Sadowska-Bartosz and Bartosz, 2014). This has been confirmed in previous experiments with mice, in which the old animals that ingested diet supplemented with appropriated amounts of antioxidants showed an improvement of immune cell functions, a better redox state and increased longevity (De la Fuente and Miquel, 2009; De la Fuente et al., 2011; Sadowska-Bartosz and Bartosz, 2014). Although there is experimental evidence showing that antioxidants such as vitamin C and E may prevent or delay the oxidative stress and the physiological impairment associated with physiological and pathological aging, there are also controversial results, especially epidemiological evidence, on the positive effect of antioxidant vitamin supplementations increasing well-being, the doses of antioxidants being one of the most relevant causes of that controversy (Viña et al., 2007; Lykkesfeldt and Poulsen, 2010; Sadowska-Bartosz and Bartosz, 2014; Hemillä, 2020). Thus, a hormetic role of dietary antioxidants, with a U-shaped dose response in the redox situation of the organism has been proposed (Calabrese et al., 2010). In fact, even in the case of vitamin C, with several studies recommending the use of high doses of this antioxidant to cure and prevent common cold infections and to prevent the onset of cancer (Verrax and Calderon, 2009), and showing that doses of 2.000 or 5.000 mg/day are well tolerated without negative effects on immune cell function (Jacob and Sotoudeh, 2002), the possible pro-oxidant role of high amounts of ascorbic acid in certain circumstances (Childs et al., 2001), advises caution with the amount of this antioxidant used in the supplementation trials. However, 1000 mg/day intake of vitamin C supplementation, accompanied by a diet rich in fruit and vegetables has been recommended for optimal health (Deruelle and Baron, 2008) and this dose showed positive effects on immune response (Wintergerst et al., 2006). Moreover, it has been shown that higher doses did not increase the leukocyte incorporation of the vitamin (Vodjani et al., 2000). The supplementation with vitamin E, which has shown controversial results depending on the doses, improved the immune functions studied in the present work using 200 mg/day (De la Fuente et al., 2008).

With respect to the adherence capacity of leukocytes and the superoxide levels of neutrophils, the increases with aging in these functions were lowered bringing their values near those of adults after vitamin C intake. Adherence of lymphocytes or phagocytes is the first event in the immune and inflammatory response and it is a function that precedes the migration (i.e. chemotaxis) of immune cells. Leukocyte adherence increases in oxidative situations such as chronological aging, premature aging or endotoxic shock, because free radicals stimulate the expression of adherence molecules (De la Fuente and Víctor, 2000; De la Fuente and Miquel, 2009). Although there are some data in which these vitamins increase leukocyte adherence (De la Fuente et al., 1998), in agreement with the present results, a decrease of the adhesion of monocytes to endothelial cells (Woollard et al., 2002) as well as the expression of adhesion molecules on these cells (Rayment et al., 2003) has been observed with vitamin C supplementation. Moreover, a decrease of adherence in neutrophils and lymphocytes from elderly men and women has been found with vitamin E supplementation (De la Fuente et al., 2008). Vitamin C and vitamin C plus E ingestion also decreased the intracellular superoxide levels of neutrophils. The ingestion of antioxidants such as vitamin E and *N*-acetylcysteine appears to slow down the age-related increase in superoxide production by neutrophils (De la Fuente et al., 2008; Arranz et al., 2008). An oral vitamin C therapy in chronic heart failure patients, with high levels of oxidative stress, decreased neutrophil superoxide anion generating capacity and concomitant oxidative stress (Ellis et al., 2000). In this respect, although ROS production is an important mechanism of microorganism destruction by phagocytes, there is evidence of a positive correlation between low levels of superoxide anion and bactericidal activity (Boxer, 1995). Nevertheless, the high levels found by us in neutrophils from elderly men and women before antioxidant intake could be harmful for immune cells and the surrounding cells and tissues (De la Fuente and Miquel, 2009; Martinez de Toda et al., 2020). Moreover, Wolach et al. (Wolach et al., 2000) showed that excessive superoxide generation had no parallel effect on bactericidal capacity. Besides, the decrease in the oxidative status of elderly women after vitamin C and E supplementation is in agreement with previous results showing a decrease of lipid peroxidation in serum, determined by the malondialdehyde (MDA) levels, in elderly women after supplementation with these antioxidants (De la Fuente et al., 1998).

Other functions such as the chemotaxis of neutrophils and

lymphocytes as well as the phagocytic capacity of neutrophils, which decreases with aging, are increased after vitamin C and vitamin C + E intake, improving their defence function. Vitamin C supplementation (2.000 mg/day) for 2 weeks restored the chemotaxis of monocytes from smokers, which was decreased with respect the non-smoker controls (Stadler et al., 2007). A significant 20% increase in neutrophil chemotaxis was obtained after four weeks of dietary supplementation with vitamin C-rich SunGold kiwifruit (Bozonet et al., 2015). With respect to the phagocytic function, in peritoneal macrophages of mice and guinea pigs, ascorbic acid was used in the phagocytosis process and thus there was a decrease in its levels during the ingestion of foreign particles (Hernanz et al., 1990). In vitro, vitamin C modulates phagocytosis activity in immune cells (Ströle et al., 2011). These results could explain the increase of phagocytosis capacity after the vitamin C supplementation. In a previous study vitamin E intake also increased chemotaxis of neutrophils and lymphocytes as well as the phagocytosis of neutrophils in elderly men and women (De la Fuente et al., 2008).

With respect to T-lymphocyte proliferation in response to mitogens and the release of IL-2 cytokine, two functions that clearly decrease with aging, the supplementation with vitamin E has shown a positive effect (Meydani et al., 2005; De la Fuente et al., 2008). The effect of Vitamin C on these functions has seldom been studied and contradictory results have been obtained, even no significant effect on proliferation (Douziech et al., 2002; Ströle et al., 2011), and have shown a dose-dependent inhibition of IL-2 producing lymphocytes upon PMA/ionomycin stimulation (Härtel et al., 2004). However, in the present work an increased proliferation of lymphocytes in response to PHA and of IL-2 release have been observed in elderly men and women after vitamin C supplementation, and the same occurs with the vitamin C and E intake. Since one important cause of the age-related impairment of lymphocyte response to mitogens is a progressively decreasing proportion of functional T cells, which could be due to excessive apoptosis, it has been suggested that one potential mechanism underlying the enhanced immune response by vitamin C may be the inhibition of leukocyte apoptosis signalling pathways that this antioxidant causes (Perez-Cruz et al., 2003).

With respect to the NK cytotoxicity against tumours, the stimulation found in the present study is in agreement with previous work using the same dosage of vitamin C (500 mg/day) (Vodjani et al., 2000). Vitamin E also improves NK activity in elderly men and women (De la Fuente et al., 2008).

Because there are data supporting the idea that immune function in aging is similar to that in inflammatory conditions (De la Fuente and Miquel, 2009; De la Fuente, 2018) and that antioxidants also have anti-inflammatory effects, they may act in this way on immune function (Härtel et al., 2004; Singh et al., 2005). Thus, vitamin C could act as anti-inflammatory inhibiting the initial expression of pro-inflammatory cytokines and also their autocrine stimulation pathway via nuclear factor kappa B (NFkB) (Cárcamo et al., 2002; Härtel et al., 2004). The anti-inflammatory effect of vitamin E, which acts decreasing the production of prostaglandins by phagocytes (Meydani et al., 2005), is also mediated via decrease of a high activation of NFkB (Wu and Meydani, 2008). The levels of NFkB expression in peritoneal leukocytes of mice are related to the oxidative and inflammatory stresses in these cells, with their function and with the span of life of the subjects (De la Fuente and Miquel, 2009; Arranz et al., 2010). Thus, a lower NFKB expression in leukocytes is associated with better immune response, redox state and longevity (Arranz et al., 2010). The activation of NFkB, a potential mediator of the inflammation and oxidative stress in immune cells, must be under tight control because adequate levels of this activation are essential for a good preserved homeostasis and functional response in immune cells (Arranz et al., 2010). In fact, certain amounts of oxidation and inflammation, two related processes, are necessary for a good immune response, but their defect or excess is associated with inappropriate immunity or immunosenescence (De la Fuente, 2018). Moreover, the controlled NFkB activation could contribute importantly to an antioxidant environment in leukocytes, which would allow their well-preserved response to stimuli (Arranz et al., 2010). Thus, the role of vitamin C and vitamin E modulating the NFkB activation levels can explain the positive effects of theses antioxidants on the immune functions studied in the present work. These vitamins can act as anti-inflammatory compounds and however increase immune activities that need an inflammatory response, such as lymphocyte proliferation.

Although the beneficial results obtained with vitamin C and vitamin C plus E supplementations could be due to their antioxidant and anti-inflammatory roles, through control of NFkB activity, other pathways such as the regulation of the hypoxia-inducible factors (HIFs) could be also involved. In fact, the interactions of vitamin C with HIFs are relevant to the function of immune cells in inflammation (Ang et al., 2018). In addition, in the positive results show by these vitamins on the functions of immune cells, especially on the lymphocyte activities, other factors could be also considered, such as their gene regulating effects (Carr and Maggini, 2017) or their action on the T lymphocyte receptor (TCR) and its intracellular signalling (Lee and Han, 2018; Peters et al., 2020).

Since an oxidative and inflammatory stress is in the base of the immunosenescence, which is involved in the rate of aging (De la Fuente and Miquel, 2009; De la Fuente, 2018; Martinez de Toda et al., 2020), the ingestion of adequate amounts of antioxidants such as vitamin C and vitamin E could regulate the immune cell functions and therefore oxi-inflamm-aging of the subjects. We know that some research questions the positive role of the ingestion of antioxidant vitamins, especially in high doses, in the organism as consequence of a possible decrease that they cause on the endogen antioxidant defences (Viña et al., 2007). Nevertheless, several studies show the positive role of supplementation with moderate levels of antioxidant vitamins (Alvarado et al., 2006; Arranz et al., 2008; De la Fuente et al., 2008; De la Fuente and Miquel, 2009; De la Fuente et al., 2011). Thus, although vitamin C (1000 mg/day) and E (400 IU/day) ingestion prevented the induction by exercise of several endogen antioxidant defences (Ristow et al., 2009), in another study vitamin C (152 mg/day) and E (50 mg/day) decreased the exercise-induced oxidative damage, without blocking the cellular adaptation (Sureda et al., 2008). Moreover, vitamin C supplementation (4 times a day in a 500 mg dose) suppressed the lipid peroxidation process during exercise (Popovic et al., 2015).

Although the results obtained are, in general, very similar in men and women, there are several interesting differences in the immune functions studied and in the effects of vitamin supplementations between genders. The idea of that the immune functions and their age-related changes are some different in male and female mammals, are becoming more evident in the last years, females showing a better immune response against infections and lower oxidation than males (Alonso-Fernandez and De la Fuente, 2011; Ostan et al., 2016). In the present study, immune functions that increase with oxidative state such as adherence and superoxide anion levels are more increased in leukocytes from elderly men than in those from women, in agreement with previous results (De la Fuente et al., 2008). However, the effects of vitamin supplementations on some functions are slightly higher in women than in men, although some effects are maintained longer after finishing supplementation in men. Thus, since there are few studies about the differences between both sexes on the effects that nutritional interventions or other strategies of lifestyle show on immune functions in aging, to consider these gender differences in the future research seem to be very necessary, as many authors have suggested (Ostan et al., 2016; Tidière et al., 2020).

Finally, if some work suggests that the improvement of immune parameters in a population with a generally good immune and nutritional status is limited (Wolvers et al., 2006), the results of the present study confirm, at least in elderly populations, the positive effects on the immune system of the supplementation used and thus, its possible role in the decrease of duration and severity of infections as was previously suggested. Moreover, these vitamin supplementations seem useful to

rejuvenate the immune system, since they bring the values of immune parameters studied closer to those of adult subjects. In prematurely aging mice the ingestion of a diet enriched with nutritional doses of antioxidants such as vitamin C, vitamin E, among others, improved the peritoneal leukocyte functions, restored their redox balance (Alvarado et al., 2006) and increased the life span of the animals (data sent to be published). Thus, since the age-related changes of immune functions such as those studied in the present work, are similar in peritoneal leukocytes of mice and in peripheral blood leukocytes of humans, and since these immune parameters are markers of health, biological age and longevity (De la Fuente and Miquel, 2009; Martinez de Toda et al., 2016), it is possible to suggest that the supplementation used in the present study could improve the quality of life and extend a healthy longevity in elderly men and women.

If the effects obtained are consequences of the antioxidant properties of vitamin C and E or if they act as physiological-redox-signalling modulators is an interesting subject for future research.

Author contributions

FA carried out the selection of participants, the interviews and the obtention of samples, as well as being one of those responsible for the design of the study. MDF and AH took part in the design, statistical analysis and discussion of results. MDF has written the manuscript. MDF, FA and AH were responsible of the final content of this manuscript. CS and CV carried out all the experiments. EDC helped in performing several of the experiments. All authors have read and approved the final manuscript.

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Declaration of competing interest

The authors declare no financial or other conflict of interests regarding the publication of this paper.

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