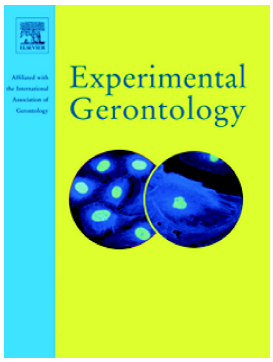

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Vitamin C and vitamin C plus E improve the immune function in the elderly

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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.

Keywords: Vitamin C; Vitamin E; Aging; Immune function; Men and Women.

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 [1-5]. 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 [2-7].

In addition, it has been shown that the competence of the immune system is an excellent marker of health [8] 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" [2,6].

Among all the many theories about how the aging process occurs, the free radical-oxidation theory is one of the most widely accepted [2,9-11]. More recently, the theory of oxidation-inflammation has been proposed [2]. 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 [12]. This oxidative-inflammatory

stress affects all cells of the organism but especially those of the homeostatic 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 [2].

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 [2,4,13,14]. An important number of studies show that the ingestion of diets with adequate levels of antioxidants such as vitamins E and C, ß-carotene, polyphenols and others, are able to retard or prevent the oxidative damage [4,15] 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 [2,4,16].

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 [2,4,17-24].

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 [2,4,25-26].

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 [27-29]. 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 [2,4,11,30]. Thus, it has been observed that low blood concentrations of vitamin C in old population are a strong predictor of mortality [22]. Although previous studies have shown clearly that vitamin E supplementation improves several immune functions in the elderly [27,28], 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 [31-33]. 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 [34]. 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 [35].

In view of the above, we conducted a study to determine the effect of vitamin C supplementation (500mg/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 α=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 [35]. 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 [18,27,37,38]. Since intakes of up to 1.000 mg/day of vitamin C show a favourable effect on immune response [18], and it has been indicated that an intake of 200 mg/day at least is needed to increase immune functions [37], 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 [27], as well as with protective effects on upper respiratory infections [38], was chosen.

2.5. Separation of blood neutrophils and lymphocytes

Peripheral blood neutrophils and lymphocytes were obtained following a method previously described [27], 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 $5x10^5$ neutrophils/ml medium and $1x10^6$ 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 [27]. 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, 1mL of whole blood (diluted 1:1 with Hank's medium) was placed in a Pasteur pipette in which 50 mg of nylon fiber was packed to a height of 1.25 cm. After 10 minutes, the effluent had drained by gravity. The results were expressed as adherence index (A.I), which was calculated as follows:

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

The chemotaxis was evaluated measuring the mobility capacity of neutrophils towards an infectious focus, using Boyden chambers with 2 compartments separated by a

polycarbonate 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 3h 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 37ºC, 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. [27] slightly modified as follows. Aliquots of 250 μl neutrophil suspension (10^6 cells/ml Hank's medium) were mixed with 250 μl NBT (1mg/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 [27].

The lymphoproliferation assay was performed using a standard method, previously used by us [27]. 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 semiautomatic 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 [27]. 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 [27].

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:

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

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.

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

Characteristics	Adult subjects	Old subjects
Number	30	44
Gender Man Woman	 15 15	 20 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 (mmHg)	90±5	100±6

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 **Figures 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.

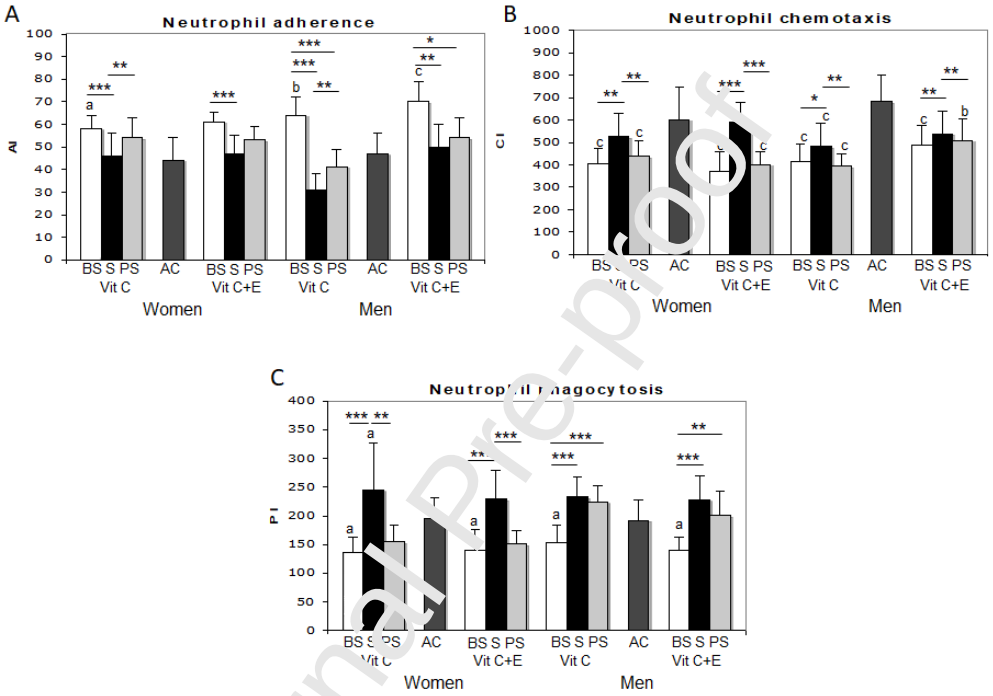

Figure 1. Neutrophil adherence (adherence index (AI): percentage of neutrophil adherence to nylon fibre) (A), chemotaxis (chemotaxis index (CI): number of neutrophils on filter) (B) and phagocytosis (phagocytic index (PI): number of latex beads/100 neutrophils) (C) capacities of cells from adult controls (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.

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 **Figure. 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.

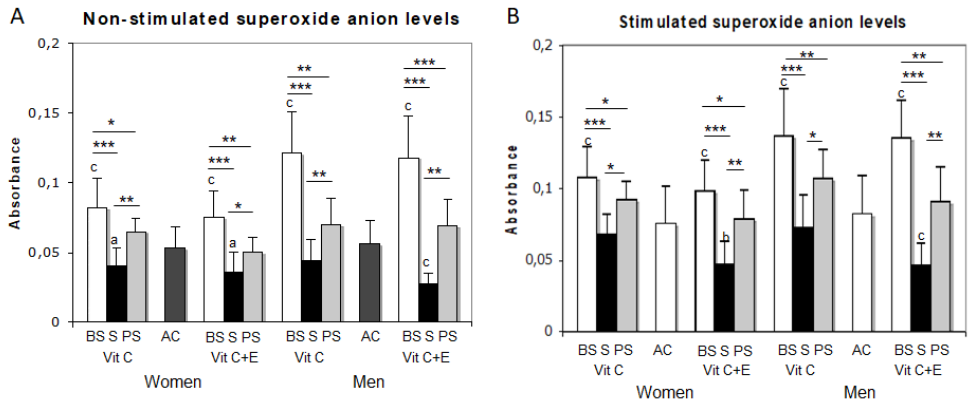

Figure 2. Superoxide anion levels in non-stimulated and stimulated samples (with latex beads) (Absorbances) of human peripheral neutrophils from adult (controls) (AC) and elderly men and women before (BS), after 3 months of vitamin C or vitamin C plus vitamin E supplementations (S) and 6 months after 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.

With respect to the lymphocyte functions studied, the results of adherence (A.I.) and chemotaxis (C.I.) are shown in **Figure 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.

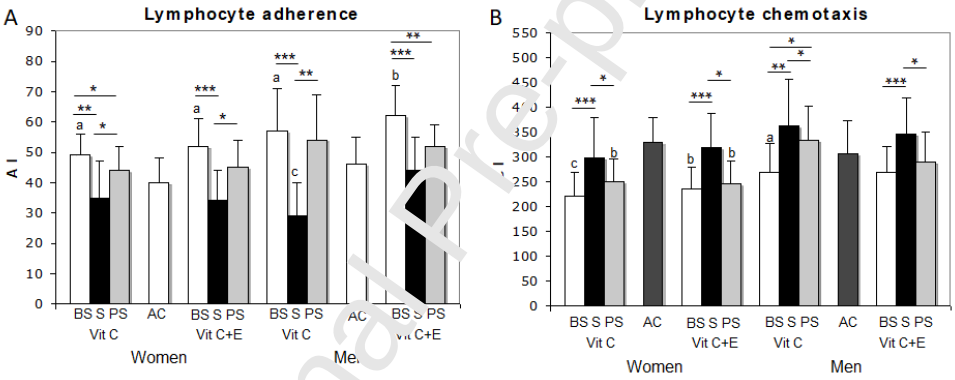

Figure 3. Lymphocyte adherence (adherence index (AI): percentage of lymphocyte adherence to nylon fibre) (A) and chemotaxis (chemotaxis index (CI): number of lymphocytes on filter) (B) capacities of cells from adult controls (AC) and elderly subjects before (BS), after 3 months of vitamin C or vitamin 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.

The lymphoproliferative capacity in response to PHA, the IL-2 release and the NK activity are shown in **Figure 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.

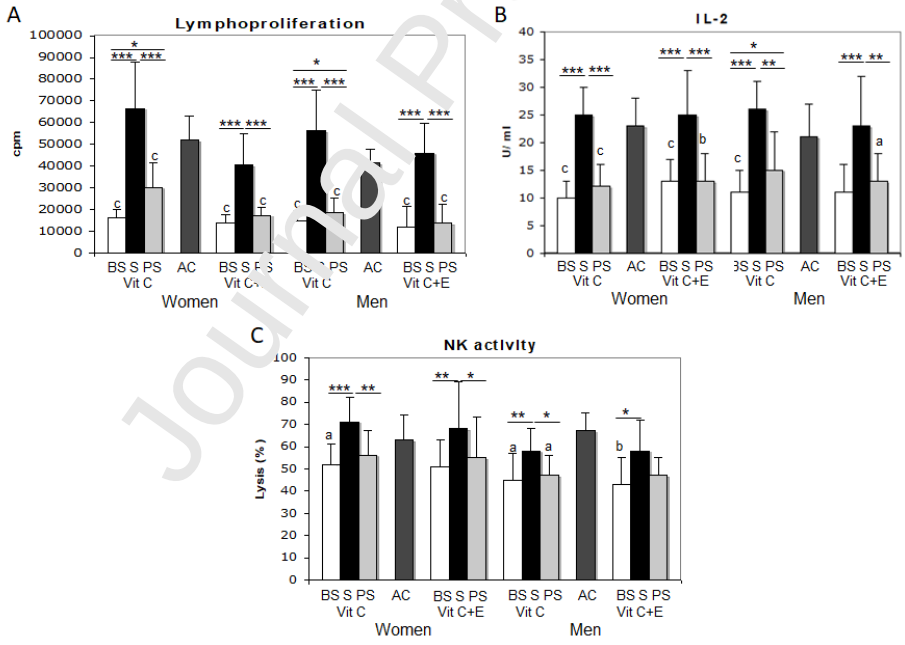

Figure 4. Proliferation in response to PHA (counts per minute: cpm) (A), IL-2 levels (U/ml) in supernatants of PHA-stimulated cultures of lymphocytes (B) and NK activity (lysis % of human tumour cells) (C) of human peripheral lymphocytes from adult controls (AC) and elderly subjects before (BS), after 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.

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 [34,39], 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 [27,39].

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 [2,5,6]. 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 [2,5,6]. However, adherence capacity, of both lymphocytes and phagocytes, increases with aging as several studies have shown [2,27]. 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 [27,40,11]. 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 [2,12], 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 [41]. 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 [2,4,41]. 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 [41-44]. Thus, a hormetic role of dietary antioxidants, with a U-shaped dose response in the redox situation of the organism has been proposed [15]. 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 [45], and showing that doses of 2.000 or 5.000 mg/day are well tolerated without negative effects on immune cell function [46], the possible pro-oxidant role of high amounts of ascorbic acid in certain circumstances [47], advises caution with the amount of this antioxidant used in the supplementation trials. However, 1000mg/day intake of vitamin C supplementation, accompanied by a diet rich in fruit and vegetables has been recommended for optimal health [48] and this dose showed positive effects on immune response [18]. Moreover, it has been shown that higher doses did not increase the leukocyte incorporation of the vitamin [49]. 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 [27].

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 [2,39]. Although there are some data in which these vitamins increase leukocyte adherence [34], in agreement with the present results, a decrease of the adhesion of monocytes to endothelial cells [50] as well as the expression of adhesion molecules on these cells [51] 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 [27]. 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 [27,40]. 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 [52]. 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 [53]. 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 [2,11]. Moreover, Wolach et al. [54] 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 [34].

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 [19]. A significant 20% increase in neutrophil chemotaxis was obtained after four weeks of dietary supplementation with vitamin C-rich SunGold kiwifruit [55]. 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 [17]. In vitro, vitamin C modulates phagocytosis activity in immune cells [56]. 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 [27].

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 [27,57]. The effect of Vitamin C on these functions has seldom been studied and contradictory results have been obtained, even no significant effect on proliferation [56,58], and have shown a dose-dependent inhibition of IL-2 producing lymphocytes upon PMA/ionomycin stimulation [59]. 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 [60].

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 (500mg/day) [49]. Vitamin E also improves NK activity in elderly men and women [27].

Because there are data supporting the idea that immune function in aging is similar to that in inflammatory conditions [2,12] and that antioxidants also have anti-inflammatory effects, they may act in this way on immune function [59,61]. 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) [59,62]. The anti-inflammatory effect of vitamin E, which acts decreasing the production of prostaglandins by phagocytes [57], is also mediated via decrease of a high activation of NFkB [63]. 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 [2,64]. Thus, a lower NFKB expression in leukocytes is associated with better immune response, redox state and longevity [64]. 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 [64]. 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 [12]. Moreover, the controlled NFkB activation could contribute importantly to an antioxidant environment in leukocytes, which would allow their well-preserved response to stimuli [64]. 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 [21]. 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 [20] or their action on the T lymphocyte receptor (TCR) and its intracellular signaling [26, 63].

Since an oxidative and inflammatory stress is in the base of the immunosenescence, which is involved in the rate of aging [2,11,12], 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 [42]. Nevertheless, several studies show the positive role of supplementation with moderate levels of antioxidant vitamins [2,4,27,31,40]. Thus, although vitamin C (1000mg/day) and E (400IU/day) ingestion prevented the induction by exercise of several endogen antioxidant defences [66], in another study vitamin C (152 mg/day) and E (50 mg/day) decreased the exercise-induced oxidative damage, without blocking the cellular adaptation [67]. Moreover, vitamin C supplementation (4 times a day in a 500mg dose) suppressed the lipid

peroxidation process during exercise [68].

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 [69,70]. 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 [27]. 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 [70,71].

Finally, if some work suggests that the improvement of immune parameters in a population with a generally good immune and nutritional status is limited [72], 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 [31] 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 [2, 6], 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.

5. 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.

6. Declarations of interest

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

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Highlights

- Vitamin C supplementation improves neutrophil and lymphocyte functions in elderly men and women.
- Supplementation with vitamin C and E restore functions of immune cells in the elderly.
- In elderly vitamin C show similar or better improvement of immune cell function than vitamin C plus E.
- The positive effects of vitamins on immunity are maintained after finishing supplementation.
- Vitamin C and E supplementation could increase healthy longevity.