IMPROVEMENTS IN BEHAVIOR AND IMMUNE FUNCTION AND INCREASED LIFESPAN OF OLD MICE COHABITATING WITH ADULT ANIMALS

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Abstract

The social environment can affect the regulatory systems, and cohabitation with sick subjects is a negative factor for the nervous and immune systems, compromising the lifespan. Nevertheless, the possible beneficial effects of a positive social environment on nervous and immune functions and longevity have not yet been studied. The aim of the present work was to analyze several behavioral and immune function parameters and lifespan in old mice after their cohabitation with adult animals. Old and adult ICR-CD1 female mice were divided into three experimental groups: adult controls, old controls and a social environment experimental group. The latter contained 2 old with 5 adult mice. After two months in these conditions, mice were submitted to a behavioral battery of tests in order to analyze their sensorimotor abilities, anxiety-like behaviors and exploratory capacities. Peritoneal leukocytes were then collected and several immune functions as well as oxidative and inflammatory stress parameters were assessed. The animals were maintained in the same conditions until natural death occurred. The results showed that old animals, after cohabitation with adult mice, presented an improvement of behavioral capacities, immune functions and a lower oxidative and inflammatory stress. Consequently, they exhibited a higher lifespan.

Keywords: Social environment, longevity, environmental strategy.

Introduction

Regulatory systems (nervous, immune and endocrine) suffer an age-related impairment, which is associated to loss of homeostasis, and hence, of the health [1]. This decline seems to be due to the establishment of chronic oxidative and inflammatory stress provoked by accumulation of oxidant and inflammatory compounds together with a decrease in antioxidant and anti-inflammatory defenses [2]. In the context of the aging of the nervous system, older individuals exhibit, as the consequence of alterations of nervous cell function, an impairment in sensorimotor abilities and lower locomotion, together with an increase in anxiety-like behavior among other characteristics [3,4]. In addition, the age-related changes in the immune system, namely immunosenescence, increase the susceptibility to suffer infectious processes, autoimmune diseases and cancers. In fact, the function capacity of the immune system has been considered a good marker of health. Moreover, several immune functions have been proposed as markers of the rate of aging of each subject, showing his/her biological age and consequently as predictors of life expectancy [5, 6].

Last decade, due to increase in the older population, there has been an increase in research related to strategies, which could slow the evolution and consequences of the aging process. Accumulating evidence suggests that maintenance of adequate health depends on life style [7]. Thus, strategies, such as nutrition or exercise, have been shown to be positive interventions capable of slowing down the aging process [8]. In humans and in other social species, the social context may profoundly influence physiological and behavioral responses [9]. Nevertheless, most studies have aimed at examining the effects of negative social environment on health. Thus, loneliness in humans and social isolation in rodents has been shown to cause behavioral abnormalities and immune system function impairments, which result in a decreased lifespan [10, 11]. In addition, in the case of rodents, the cohabitation of healthy mice with sick individuals resulted in negative influences on the behavior and immunity of the former. These included increased anxiety-like behaviors and impaired phagocytic process, evaluated in blood neutrophils and peritoneal macrophages [12]. In healthy animals, the induction of scratching and dermatitis after cohabitating with individuals with this pathology were observed [13]. In contrast, although the maintenance of positive social interactions seems to be related to increased lifespan [14], the beneficial effects of these on the nervous and immune systems, in the context of aging, have not yet been studied. Therefore, the aim of the present work was to study the possible positive effects on several behavioral and immune

function parameters of chronologically old mice after cohabitation with adult animals, and if these increase the lifespan of old mice.

Methods

Animals

We used 16 old (21±1 months) and 28 adult (9±1 months) female ICR-CD1 mice (Janvier, France). All mice were housed 7-8 per cage and maintained in standard laboratory animal conditions for pathogens, temperature (22±2ºC) and humidity (50-60%), on a 12/12h reversed light/dark cycle (lights on at 20:00h) to avoid circadian interferences. Mice had access to tap water and standard pellets *ad libitum*. Diet was in accordance with the recommendations of the American Institute of Nutrition for laboratory animals (A04 diet from Panlab S.L., Barcelona, Spain). The protocol was approved by the Experimental Animal Committee of the Complutense University of Madrid (Spain). Animals were treated according to the guidelines of the European Community Council Directives 2010/63/EU.

Experimental design

After one week of adaptation to room conditions, mice were divided into the following groups: adult control group (AC; N=8), old control group (OC; N=8) and four cages namely social environment cages, each one containing 2 old mice (old social environment, OSE) and 5 adult mice (adult social environment, ASE). After two months in these environments, mice were submitted to a battery of behavioral tests in order to evaluate sensorimotor abilities, anxiety-like behaviors and exploratory capacities. Peritoneal leukocytes were then extracted and several immune functions as well as oxidative and inflammatory stress parameters were assessed. Then, animals were maintained in these living conditions, in order to analyze their lifespan. In the case of ASE mice, they were maintained in the same cage and under the same conditions after the death of OSE animals.

Behavioral tests

Behavioral tests were carried out following methods used in previous studies [15, 16].

Sensorimotor abilities

Visual placing and hindlimb extensor reflexes

Visual placing and hindlimb extensor reflexes were performed following the protocol previously described [15]. Complete extension of the forelimbs as well as complete extension of the hindlimbs were considered a positive response.

Wood rod test

With the objective to evaluate motor coordination, a wood rod test was performed following the protocol previously described [15]. The time of latency (in seconds) to leave the starting segment and the total number of crossed segments were measured.

Tightrope test

The tightrope test, which evaluates motor coordination, muscular vigor and traction [15, 17], was performed. Motor coordination was evaluated by the latency to leave the starting segment (in seconds) and by the total number of crossed segments. Muscular vigor was determined by the percentage of mice falling off the rope and the latency of fall (in seconds). Finally, traction was analyzed by observing the different parts of the body that mice used to remain hanging (forelimbs, hindlimbs and tail) and by the percentage of mice displaying the maximum traction capacity (employing the three parts of the body).

Exploratory and anxiety-like behavioral tests

Holeboard test

In order to evaluate exploratory and anxiety-like behaviors, the holeboard test was performed following the previously described protocol [15]. The parameters recorded for “non-goal directed behavior” and related with horizontal activity were: total locomotion, percentages of inner locomotion and external locomotion. The rearing (total number and time in seconds) was the parameter analyzed related to vertical activity. Furthermore, the head-dipping (total number and time in seconds) was evaluated as “goal-directed behavior”. Finally, self-grooming and freezing behaviors were also recorded.

T-maze test

A T-maze test was used, following the previously described protocol, to analyze spontaneous horizontal exploration [18]. This behavior included: time (in seconds) to cross the intersection of the maze with both hindlimbs and total time (in seconds) spent to explore the entire maze.

Elevated plus maze

The elevated plus maze, a typical test to evaluate anxiety-like behaviors, was performed following the protocol previously described [15,19]. The total number of entries in open arms as well as in closed arms was recorded. Total grooming numbers were also registered. Finally, the percentages of time spent in open and in closed arms as well as in the platform were calculated.

Immune function parameters

Immune function parameters were evaluated in peritoneal leukocytes. The collection of these was carried out following a method previously described [20].

Macrophage phagocytosis

The phagocytic capacity was evaluated following a method previously described [6]. Aliquots of peritoneal suspensions adjusted to 5 x 10^5 cells/mL Hank´s solution were incubated on migration inhibition factor plates for 40 min. To the adherent monolayer, aliquots of latex beads were added. After 30 min of incubation, the plates were washed, fixed and stained and the number of particles ingested by 100 macrophages was counted by optical microscopy (100x), being expressed as a phagocytic index. The percentages of macrophages that ingested latex beads were also counted and expressed as phagocytic efficiency.

Chemotaxis

The chemotaxis assays were performed according a method previously described [6]. Aliquots of peritoneal suspensions adjusted to 5 x 10^5 macrophages/mL Hank´s or 5 x 10^5 lymphocytes/mL Hank´s, were deposited in the upper compartment of the chambers and the chemo-attractant peptide f-met-leu-phe was placed in the lower compartment. After 3h

incubation, the filters were fixed and stained, and the chemotaxis index (C.I.) was determined by counting the total number of macrophages or lymphocytes in one third of the lower face of the filters.

Lymphoproliferation assay

The lymphoproliferation was assessed following a method previously described [6]. Resting lymphoproliferation as well as that in response to lipopolysaccharide (LPS, Escherichia coli, 055:B5; Sigma-Aldrich) were evaluated. Aliquots of peritoneal suspensions, adjusted to 10^6 lymphocytes/mL in complete medium containing 1640 RPMI supplemented with gentamicin (10 mg/mL) and 10% heat-inactivated fetal calf serum, were dispensed into 96-well plates. Complete medium or LPS (1 μg/mL) were added. After 48h of incubation, 100 μL of culture supernatants was collected for cytokine measurements. 0.5 μCi [^{3}H] thymidine were then added to each well and the medium was renewed, followed by another 24 h incubation. Finally, cells were harvested in a semi-automatic harvester (Skatron Instruments, Norway) and thymidine uptake was measured in a beta counter (LKB, Uppsala, Sweden) for 1 min. In the case of resting lymphoproliferation, the results were expressed as counts per minute (c.p.m.). Lymphoproliferative response to LPS was expressed as a percentage (stimulation index), with 100% being the thymidine uptake c.p.m. in control wells (without mitogen).

Cytokine measurements

Cytokine levels, including the pro-inflammatory cytokines IL-1 beta as well as IL-6 and the anti-inflammatory cytokine IL-10 were measured simultaneously by luminometry using a mouse cytokine/chemokine panel (Milliplex MAP kit, Millipore). Briefly, 25 μL of standard, control or samples were added to the appropriate wells. Later, 25 μL of premixed beads were added to each well and incubated overnight at 4ºC with shaking. After two washes, 25 μL of detection antibody were added to each well and incubated for 1h at room temperature and then treated with streptavidin-phycoerythrin (25 μL) for 30 min at room temperature. Finally, the beads were re-suspended in 150 μL of sheath fluid and the plate was read using a luminometer. The results were expressed as pg/mL. Concentrations as low as 5.4 pg/mL for IL-1beta, 1.1 pg/mL for IL-6 and 2.0 pg/mL for IL-10, were detected.

Natural killer activity

Natural killer activity was measured following a method previously described [6]. Briefly, target cells (YAC-1 cells) were seeded in 96-well U-bottom culture plates at a concentration of 10^4 cells/well in 1640 RPMI without phenol red and 10^5 peritoneal leukocytes/well (effector cells) were added, being the effector/target cell ratio 10/1. LDH enzymatic activity was measured in 50 µL/well supernatant by adding the enzyme substrate and recording absorbance at 490 nm. Three kinds of control measurements were performed: target spontaneous release, target maximum release, and effector spontaneous release. To determine the percentage of lysis of target cells, the following equation was used: % lysis=[(E-ES-TS)/(M-TS)]x100, where E is the mean of absorbance values in the presence of effector and target cells; ES the mean of absorbance values of effector cells incubated alone; TS the mean of absorbance values of target cells incubated alone and M is the mean of maximum absorbance values after incubation of target cells with lysis solution.

Oxidative stress parameters

Catalase activity

Catalase activity was determined following a method previously described [21]. The enzymatic assay was carried out spectrophotometrically for 80 s at 240 nm throughout the decomposition of H_2O_2 (14 mM in phosphate buffer) into $H_2O + O_2$. The results were expressed as International Units (IU) of enzymatic activity per 10^6 peritoneal leukocytes.

Glutathione content

Both oxidized (GSSG) and reduced (GSH) forms of glutathione, were determined using a fluorimeter as previously described [22], adapted to 96-well plates. 1 mL of the peritoneal suspension (10^6 cells/mL Hank´s solution) was centrifuged and pelleted cells were re-suspended in phosphate buffer containing EDTA (0.1 M, pH 8). Then, samples were sonicated, and after the addition of 5 µL of $HClO_4$ (60%), they were centrifuged. 10 µL of supernatants of immune cells were dispensed into two 96-well black plates, one for each glutathione form. For GSSG measurement, 8 µL of N-ethylmaleimide (NEM, 0.04 M) was added to each well and incubated at room temperature for 30 min in the dark. Then, 182 µL of NaOH (0.1 N) and 20 µL of OPT (1mg/mL in methanol) were incorporated and the plate was incubated for 15 min under the same conditions. The fluorescence emitted by each well was measured at 350 nm excitation and

420 nm emission and the results were expressed as nmol/10^6 peritoneal leukocytes. For the measurement of GSH content, 190 μL of phosphate buffer with EDTA and 20 μL of OPT were added to the 10 μL of cell supernatants dispensed in the wells. The plate was incubated for 15 min under the same conditions, and the fluorescence emitted by each well was measured using the same wave-lengths. The results were expressed as nmol GSH/10^6 peritoneal leukocytes.

Xanthine oxidase activity

Xanthine oxidase (XO) activity was assayed in total peritoneal leukocytes using a commercial kit (A-22182 Amplex Red Xanthine/Xanthine Oxidase Assay Kit, Molecular Probes, Paisley, UK). Aliquots of total leukocytes adjusted to 10^6/mL were lysated in potassium phosphate buffer (0.05M, pH 7.4) containing EDTA (0.1M, pH 7.4; Sigma-Aldrich) and dithiothreitol (DTT, 0.5 mM, pH 7.4; Sigma-Aldrich). 50 μL of the lysate were incubated with 50 μL working solution of Amplex Red reagent (100 μM) containing HRP (0.4 U/ml) and xanthine (200 μM). After 30 min of incubation at 37ºC, the fluorescence was measured in a microplate reader (Fluostar Optima, BMG Labtech, Biomedal, Spain) using excitation and emission detection at 530 and 595 nm, respectively. The XO (10 mU/mL) supplied in the kit was used as the standard, and XO activity was measured by comparing the fluorescence of samples with that of standards. The results were expressed as units (U) of enzymatic activity per 10^6 leukocytes.

Lifespan

In order to evaluate the possible effects of cohabitation on the longevity, all experimental groups were housed in the same conditions until their natural death, which was recorded to obtain the mean lifespan.

Statistical analysis

The data were expressed as the mean±standard deviation of the values. Statistics were performed using SPSS version 21.0 (Chicago, IL, USA). The normality of the samples was tested by Kolmogorov-Smirnov test. For qualitative data, the Chi-square test was used. In the case of lifespan, the Kaplan-Maier test was used. The data were statistically evaluated by Student´s t tests, $p<0.05$ being taken as the minimum significance level.

Results

Cohabitation with chronologically adult mice improves sensorimotor and exploratory capacities as well as decreases anxiety-like behaviors in chronologically old mice

Results corresponding to sensorimotor capacities as well as exploratory and anxiety-like-behaviors are summarized in Table 1.

Sensorimotor abilities, and in particular muscular vigor, decrease with aging [15,19]. This muscular vigor was impaired in OC mice in comparison to AC animals. In fact, the percentages of mice falling off the rope in the tightrope test were higher in OC mice than AC animals ($p<0.01$) (Table 1). Nevertheless, OSE animals, after cohabitation with adult mice, had lower percentages of this parameter in comparison to OC mice ($p<0.01$), the values reaching those obtained by the AC group. However, in the case of ASE mice, the percentages were higher than their AC littermates ($p<0.05$). Regarding latency to leave the starting segment in the wood rod test, a parameter that evaluates motor coordination [15], a higher value was observed in OC animals than AC mice ($p<0.01$) (Table 1). In the case of maximum traction, evaluated in the tightrope test, OC, OSE and ASE mice presented lower values than the AC group ($p<0.01$, $p<0.05$ and $p<0.01$, respectively) (Table 1).

With regards to the exploratory behavior, evaluated in the holeboard test, the percentage of inner locomotion (Table 1), which represents the horizontal activity [15], was lower in the OC group than in AC mice ($p<0.05$). However, OSE mice exhibited a higher percentage of inner locomotion in comparison to their OC littermates ($p<0.05$), reaching the values obtained by AC mice. In addition, with respect to total number and time of rearing (Table 1), which represent a vertical exploration [15], the OC, OSE and ASE groups presented lower values in comparison to the AC group ($p<0.05$).

With respect to the time spent exploring the entire T-maze, OC animals exhibited higher values than the AC group ($p<0.05$) (Table 1). Finally, with respect to goal-directed behavior, the

total number of head-dipping (Table 1), which was lower in OC than in their AC counterparts ($p<0.01$), showed higher values in OSE mice in comparison to their OC littermates ($p<0.01$), and similar to those obtained in AC animals ($p<0.01$). Similarly, in the total time of head-dipping (Table 1), OC mice showed lower values than AC mice ($p<0.001$), but OSE animals presented higher ones in comparison to the OC group ($p<0.001$). Thus, the cohabitation of old with adult mice may improve exploratory capacities in the first. Nevertheless, ASE animals seem to have these capacities impaired.

Regarding anxiety-like behaviors, which were evaluated in an elevated plus maze, OC mice showed lower percentages of time spent in open arms than AC animals ($p<0.05$) (Table 1). Nevertheless, the OSE group exhibited higher values in this parameter than OC mice ($p<0.01$), these being similar to those obtained in the AC group. These results could indicate lower anxiety levels in old animals after cohabitation. However, the ASE group presented lower values for this parameter in comparison to AC mice ($p<0.05$). In relation to total grooming events (Table 1), evaluated in the holeboard test, a behavior related to increased anxiety levels, OC mice presented higher values in comparison to OSE animals ($p<0.05$), although the ASE group showed higher values of this parameter than the AC group ($p<0.01$). Similarly, for time of grooming (Table 1), OC mice had higher values of this parameter than AC animals ($p<0.05$) and lower values were observed in OSE mice ($p<0.05$). The ASE group presented higher times of grooming with respect to AC mice ($p<0.05$). In the case of percentage of external locomotion analyzed in the holeboard test, a parameter also related to high anxiety levels, OC mice showed higher values than the AC group ($p<0.01$), but OSE mice presented lower values than the OC group ($p<0.01$). Thus, these results may indicate a decrease of anxiety levels in old mice after cohabitation with adults. However, in adult mice this anxiety increased after cohabitation with old animals.

Cohabitation with chronologically adult mice improves immune functions in old mice

Results corresponding to immune function parameters evaluated in peritoneal leukocytes from OC, OSE, ASE and AC mice are shown in Figure 1.

All immune function parameters evaluated in peritoneal leukocytes from OC mice were lower than those of AC animals ($p<0.001$). This fact typically occurs with aging [2,6]. Nevertheless, OSE mice presented higher values in all immune parameters than their OC counterparts ($p<0.001$), reaching in most immune parameters values similar to those obtained in AC mice. However, ASE animals exhibited lower values in all immune parameters evaluated in comparison to their AC littermates ($p<0.001$). Thus, cohabitation with adult mice improves the immune functions in old animals, but this social environment impairs the immune function in adult individuals.

Cohabitation with chronologically adult mice decreases oxidative and inflammatory stress in peritoneal leukocytes from chronologically old mice

Results related to oxidative and inflammatory parameters are presented in Figures 2 and 3. As parameters of oxidative stress, we evaluated several antioxidant defenses, such as catalase activity and GSH contents as well as oxidants, such as XO activity and GSSG levels. The GSSG/GSH ratios, as an oxidative stress index, were also considered. To study the inflammatory stress, the release of pro-inflammatory cytokines such as IL-1 and IL-6 as well as the anti-inflammatory cytokine IL-10 were measured in culture supernatants of resting lymphoproliferation. The resting lymphoproliferation was also analyzed.

Oxidative stress parameters

The results of antioxidant defenses are shown in Figure 2. Regarding catalase activity (Figure 2A), which was lower in OC animals than in the AC group ($p<0.001$), the values were higher in OSE mice in comparison to their OC littermates ($p<0.001$), reaching values obtained by the AC group. However, ASE mice presented lower values of this parameter than AC animals ($p<0.001$). In the case of GSH contents, no differences were observed between experimental groups. The values obtained were: 33±4, 31±4, 31±4 and 62±28 nmol GSH/10^6 peritoneal leukocytes from OC, OSE, ASE and AC mice, respectively.

The XO activity (Figure 2B), a marker of oxidation, showed values higher in OC animals than in AC mice ($p<0.01$), whereas OSE mice presented lower activities than those in the OC group ($p<0.05$), reaching the values obtained by AC mice. Nevertheless, the ASE group

had higher values than AC mice ($p<0.05$). Similarly, GSSG levels, which were higher in OC animals than in AC mice ($p<0.001$), were lower in OSE mice in comparison to their OC group ($p<0.01$) (Figure 2C). Finally, in the case of GSSG/GSH ratios (Figure 2D), with values higher in OC animals than in AC mice ($p<0.001$), these were lower in OSE mice than in the OC group ($p<0.001$). Again, the ASE mice showed higher values of these ratios than AC animals ($p<0.001$).

Inflammatory stress parameters

The results of inflammatory stress parameters are shown in Figure 3. Regarding resting lymphoproliferation (Figure 3A), a parameter with values higher in OC mice than in the AC group ($p<0.001$), in OSE animals showed lower values in comparison to their OC littermates ($p<0.001$), reaching similar values to those obtained by AC mice. Nevertheless, ASE animals presented higher resting lymphoproliferation than AC mice ($p<0.001$). Similarly, in the values of pro-inflammatory cytokine IL-1beta (Figure 3B), which were higher in OC mice than in AC animals ($p<0.01$), OSE mice presented lower values in comparison to those in the OC group ($p<0.05$). Furthermore, ASE mice had higher values of this cytokine with respect to AC animals ($p<0.01$). In the case of IL-6 levels (Figure 3C), with values also higher in the OC group than in AC animals ($p<0.001$), OSE mice showed lower values than OC ($p<0.001$). Finally, with regards to the anti-inflammatory cytokine IL-10 (Figure 3D), with OC showing lower values than AC mice ($p<0.01$), the OSE group had higher values of this cytokine than the OC mice ($p<0.01$). Thus, the cohabitation of old mice with adult mice seems to decrease their oxidative/inflammatory stress, but it is increased in adult animals.

Cohabitation with adult mice increases lifespan in chronologically old mice

As shown in Figure 4, OSE mice exhibited higher lifespans ($p<0.001$) than their OC counterparts. No differences were observed in ASE mice with respect to AC animals.

Discussion

This work has been the first to analyze the effects on several parameters of behavior, immune function and redox state, of cohabitation of old mice with adult mice. The benefits of this cohabitation on the nervous and the immune functions in these old animals as well as their increased lifespan were observed.

A common feature of aging is the decline in motor function and cognitive abilities, which are associated with a lower quality of life [3,4]. A simple way to evaluate the nervous function in mice is by performing behavioral tests [23]. For this reason, all animals of the present work were submitted to a battery of behavioral tests in order to analyze their sensorimotor and exploration abilities, as well as their anxiety-like behaviors. Several previous reports have described an age-related decline in these capacities [3,4,24,25]. This fact has also been shown by the old control mice (OC) of the present study. However, old mice that cohabited with adult mice (OSE mice) exhibited higher sensorimotor abilities such as muscular vigor, as well as better exploration ability. Furthermore, these animals showed lower anxiety-like behaviors than OC mice, results that may indicate a slowing down of nervous system aging. In this context, although this work is the first that has analyzed the beneficial effects of this type of cohabitation on nervous function, other strategies that suppose a new positive social network, such as environmental enrichment, have shown similar results. In fact, old mice living in a social and physical active environment (classical environmental enrichment protocol) presented a similar improvement, showing increased sensorimotor abilities together with decreased anxiety-like behaviors [26]. Furthermore, a previous study has also suggested that the development of positive social networks can be effective in avoiding cognitive decline [27]. Moreover, older adults who are more socially active experience less decline in cognitive abilities [28,29]. These results appear to be in accord with those of the present study. Thus, the age-related impairment in nervous system function seems to be avoided with this kind of cohabitation.

In the immune system, the innate immunity, which supposes the first line of defense against viral and bacterial infections [30], is impaired in old individuals [6,31], as exhibited by the OC mice of the present study. However, peritoneal leukocytes from OSE mice showed a higher phagocytosis, chemotaxis of macrophages and lymphocytes as well as NK activity than the OC group, reaching similar values to those obtained in the adult control group (AC). It is known that rodents living in a positive social context present faster wound healing [32], a

process intimately related to a good innate immunity. Moreover, older adults with a positive social context showed greater natural killer cell activity in blood [33]. In agreement with these results, a previous report has shown that living in an enriched environment supposes an improvement in this immunity [34]. With respect to the proliferative response to lymphocytes in the presence of LPS, an immune function of acquired immunity, OC mice showed lower values of this parameter than AC, as many studies have shown [6,18]. Nevertheless, OSE mice had a higher proliferative response to LPS in comparison to OC mice, reaching values presented by the AC group. Similar results have been described in mice that live in an enriched environment [34]. Thus, the cohabitation with adult mice supposes an improvement of immunity (both innate and acquired) in chronologically old mice. In this context, previous reports have found a strong association between positive social context and health, especially in older individuals [14,35]. An appropriate immune function is a marker of health [5], but with advancing age, immunosenescence appears [30]. As a consequence of this there is an increase in vulnerability and susceptibility to infections, autoimmune diseases and cancers [36]. The social environmental strategy used in the present study could be appropriate to slow down the establishment of immunosenescence, thus preserving health and avoiding age-related diseases.

The oxidative and inflammatory stress associated with aging [2,37] occurred in the immune cells of OC mice studied in the present work. However, OSE mice exhibited higher antioxidant defenses (catalase activity as well as reduced glutathione contents) together with lower oxidants (xanthine oxidase activity and oxidized glutathione contents) than those in the OC group. These results may indicate a lower oxidative stress, which seems to be due to cohabitation. In fact, the GSSG/GSH ratio, a redox marker, was lower in OSE mice than their OC counterparts. Similar results have been shown using other environmental strategies, such as environmental enrichment [8, 34]. Furthermore, in the context of inflammatory stress, OSE mice had lower levels of pro-inflammatory cytokines, such as IL-1 beta and IL-6, released by basal cultures of peritoneal leukocytes. Besides, this group exhibited higher levels of IL-10, an anti-inflammatory cytokine, seeming to indicate that these animals present higher inflammatory control than their OC counterparts. Since a sterile inflammation is typical of aging [36, 38, 39], the adequate inflammatory/anti-inflammatory balance in OSE animals could represent a decrease in this type of inflammation. In fact, while the OC group had higher resting lymphoproliferation, a function related to inflammation, than AC mice, the OSE animals presented lower values than the OC group. A previous study has described a similar control of inflammation in individuals with a positive social context, showing an inverse correlation between this social environment and IL-6 levels [40]. Thus, the lower IL-6 levels observed in supernatants of resting cultures of peritoneal leukocytes from OSE mice could indicate the

existence of positive social interactions in this cohabitation. This could be one of the possible reasons for the improvement observed in this experimental group. Furthermore, the oxidant/inflammatory-antioxidant/anti-inflammatory balance is critical for immune cell function, being associated with the appearance of immunosenescence [2]. Thus, the improvement in this balance, shown by OSE mice after cohabitation, could be the basis for slowing down the establishment of their immunosenescence.

In addition, chronologically adult mice that cohabited with old animals (ASE) had an altered behavioral response, showing lower traction, vertical activity and goal-directed behavior than their AC counterparts. Furthermore, all immune function parameters evaluated in peritoneal leukocytes from these animals showed lower values than the AC group, reaching the values shown by OC mice, indicating a premature immunosenescence due to cohabitation. In addition, peritoneal leukocytes from the ASE group had lower antioxidant defenses (catalase activity) together with higher oxidant compounds (xanthine oxidase activity and GSSG/GSH ratio) as well as higher IL-1 beta levels than their AC group. Since an oxidative and inflammatory stress has been related to aging [2, 37] as commented above, these results seem to show the establishment of oxi-inflamm-aging in adults due to cohabitation, which may be the reason for their premature immunosenescence. Furthermore, these results could be due to psychological stress produced by the cohabitation with chronologically old mice. In fact, previous studies have described that psychological stress produced changes in behavior as well as a decline of immune function [41, 42]. Another report has determined that cohabitation of healthy adult animals with a sick partner can also provoke behavioral abnormalities as well as immunosuppression due to the psychological stress that appears in the healthy individuals [12].

The mechanisms that underlie the possible chronic psychological stress establishment in ASE mice due to this cohabitation are unknown. However, this stress could be due to multiple factors, such as the visual, olfactory and auditory perception of the presence of old mice. In fact, changes in behavior, odor and vocalizations have been observed in old animals [43, 44, 45, 46]. Other possible mechanisms implicated in this psychological stress establishment could be the ingestion of fecal bolei, which may alter the microbiota of the ASE group [47]. Nevertheless, further experiments are needed in order to clarify these possible processes.

Since several immune functions evaluated in the present work have been proposed as markers of the rate of aging and as predictors of life expectancy [6], the premature

immunosenescence observed in the ASE group could result in a shorter lifespan. Unexpectedly, these animals did not show a lesser longevity, a result that could be due to the development of hormetic mechanisms as a consequence of the mild- stress caused by cohabitation. In fact, several previous studies have described that the exposure to mild-stress could generate a beneficial long-term effect, leading even to the development of a resilience capacity, and thus, causing no negative effects on the next exposure to a similar stress [9, 48, 49]. However, OSE mice exhibited longer lifespan, which may be due to the improvement observed in the immune function parameters. In this sense, previous studies have described the beneficial effects of social environment on lifespan [14, 35, 50]. Thus, although with respect to behavior and immunity, this strategy seems to have bi-directional effects (positive in the case of old mice and negative in case of the adult animals), as far as longevity is concerned, only old mice are affected, increasing their mean lifespan.

In conclusion, the present work suggests that cohabitation of old mice with adult animals causes an improvement of several behavioral capacities and immune functions, slowing down the establishment of oxi-inflamm-aging. This improvement is turned into an increased lifespan. Although this environmental strategy seems to have negative effects on chronologically adult mice, which show a nervous and immune function impairment, it does not affect their lifespan. Since this work constitutes a first approach to this subject, further research is needed in order to corroborate both the beneficial and prejudicial effects of this environmental strategy. Moreover, the use of a social strategy with other characteristics could also reduce the negative effects observed in chronologically adult animals.

Conflict of interest

The authors have no conflict of interests to declare.

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Tables

Table 1. Sensorimotor abilities, exploratory capacities and anxiety-like behaviors.

	OC	**OSE**	**ASE**	**AC**
Weight (g)	40±8	48±6	50±5	45±6
Visual placing reflex				
% Mice showing this response	100	100	100	100
Hindlimb extensor reflex				
% Mice showing this response	100	100	100	100
Wood rod test				
Motor coordination				
Latency to leave the starting segment (s)	16±3 ##	9±6	5±3	2±1
Total number of crossing segments	4±1	4±0	4±0	4±1
Tightrope test				
Motor coordination				
Latency to leave the starting segment (s)	16±5	18±9	12±3	13±5
Total number of crossing segments	2±1	2±1	2±2	3±1
Muscular vigor				
% Mice falling off the rope	80 ##	33 **	57 #	30
Latency to fall (s)	15±5	20±4	16±4	28±2
Traction				
Maximum	40 ##	50 #	33 ##	80
Elevated Plus Maze				
Anxiety-like behavior				

Total number of entries in open arms	8±2	7±3	5±3	6±2
% Time in open arms	13±3 #	23±4 **	13±3 #	20±3
Total number of entries in closed arms	13±3	9±1	7±3	10±3
% Time in closed arms	46±12	44±3	45±10	37±7
% Time in central platform	42±8	33±9	42±13	43±6
Holeboard test				
Non-goal directed behavior				
Vertical activity				
Total number of rearings	2±1 #	3±1 #	2±1 #	6±1
Time of rearing (s)	2±1 #	3±1 #	2±1 #	6±2
Horizontal activity				
Total locomotion	195±57	270±78	314±60	281±68
% inner locomotion	7±2 #	12±1 *	11±3	12±2
% external locomotion	70±7 ##	53±7 **	54±5	52±4
Goal directed behavior				
Total number of head-dippings	14±4 ##	21±4 **	14±4 ##	23±4
Total time of head-dipping (s)	29±8 ###	74±12 ***	39±19	60±6
Self-grooming and -freezing behaviors				
Total number of grooming	6±3 #	2±1 *	5±2 ##	1±1
Time of grooming (s)	5±3 #	2±1 *	5±2 #	2±1
Total number of freezings	1±1	0	2±1	0
Time of freezings (s)	1±1	0	3±1	0
T-maze test				
Horizontal activity				

Time for crossing the intersection of maze (s)	11±4	9±3	5±1	5±2
Time spent to explore the entire maze (s)	25±6 #	19±4	14±3	15±3

Each value represents the mean ± standard deviation of 8 values corresponding to this number of animals. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ with respect to OC mice; # $p<0.05$, ## $p<0.01$ and ### $p<0.001$ with respect to AC mice. OC, old controls; OSE, old social environment; ASE, adult social environment and AC, adult controls.

Figure legends

Figure 1. Immune functions. Phagocytic index (A), Phagocytic efficiency (%) (B), macrophage chemotaxis (C), lymphocyte chemotaxis (D), Natural Killer activity (% of lysis) (E) and lymphoproliferative response to LPS (% stimulation) (F), evaluated in peritoneal leukocytes from OC, OSE, ASE and AC mice. Each column represents the mean ± standard deviation of 8 values corresponding to this number of animals, and each value being the mean of duplicate or triplicate assays. *** $p<0.001$ with respect to OC group; ### $p<0.001$ with respect to AC mice. OC: old controls; OSE: old social environment; ASE: adult social environment; AC: adult controls.

Figure 2. Oxidative stress parameters. Catalase activity (IU CAT/10^6 peritoneal leukocytes) (A), xanthine oxidase activity (U XO/10^6 peritoneal leukocytes) (B), GSSG contents (nmol GSSG/10^6 peritoneal leukocytes) (C), GSSG/GSH ratios (D), evaluated in peritoneal leukocytes from OC, OSE, ASE and AC mice. Each column represents the mean ± standard error of 8 values corresponding to this number of animals, and each value being the mean of duplicate or triplicate assays. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ with respect to OC mice; # $p<0.05$ and ### $p<0.001$ with respect to AC animals. OC: old controls; OSE: old social environment; ASE: adult social environment; AC: adult controls.

Figure 3. Inflammatory stress parameters. Resting lymphoproliferation (c.p.m.) (A), IL-1 beta (B), IL-6 (C) and IL-10 (D) concentrations (pg/mL) released in resting lymphoproliferation evaluated in peritoneal leukocytes from OC, OSE, ASE and AC mice. Each column represents the mean ± standard error of 8 values corresponding to this number of animals, and each value being the mean of duplicate or triplicate assays. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ with respect to OC mice; ## $p<0.01$ and ### $p<0.001$ with respect to AC animals. OC: old controls; OSE: old social environment; ASE: adult social environment; AC: adult controls.

Figure 4. Accumulated survival (in weeks) in OC, OSE, ASE and AC mice (N=8 per group) evaluated until the natural death of the animals. *** $p<0.001$ with respect to OC mice. The arrow represents the start of cohabitation. OC: old controls; OSE: old social environment; ASE: adult social environment; AC: adult controls.

Figure 1

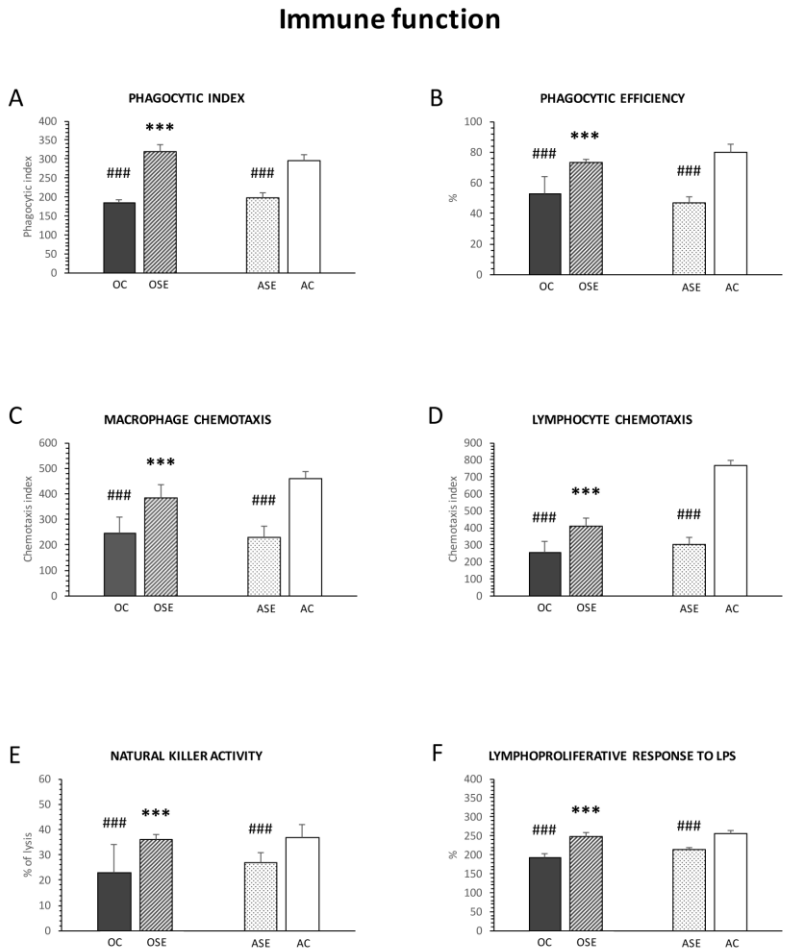

Figure 2

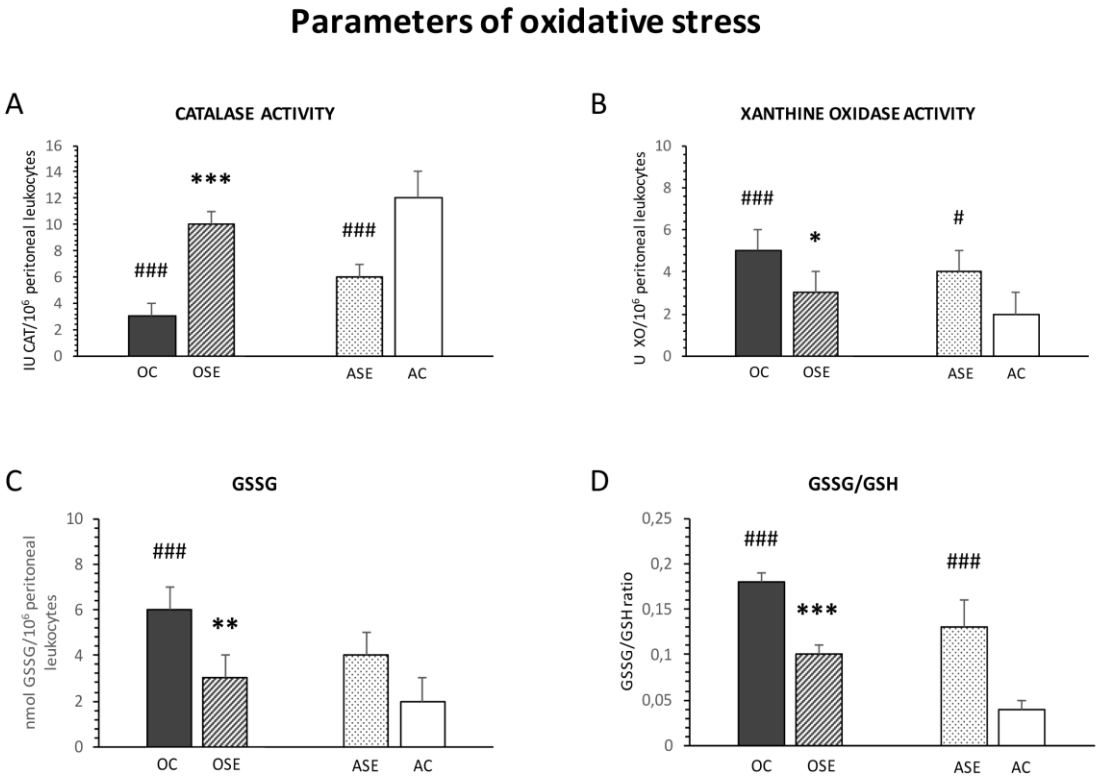

Figure 3

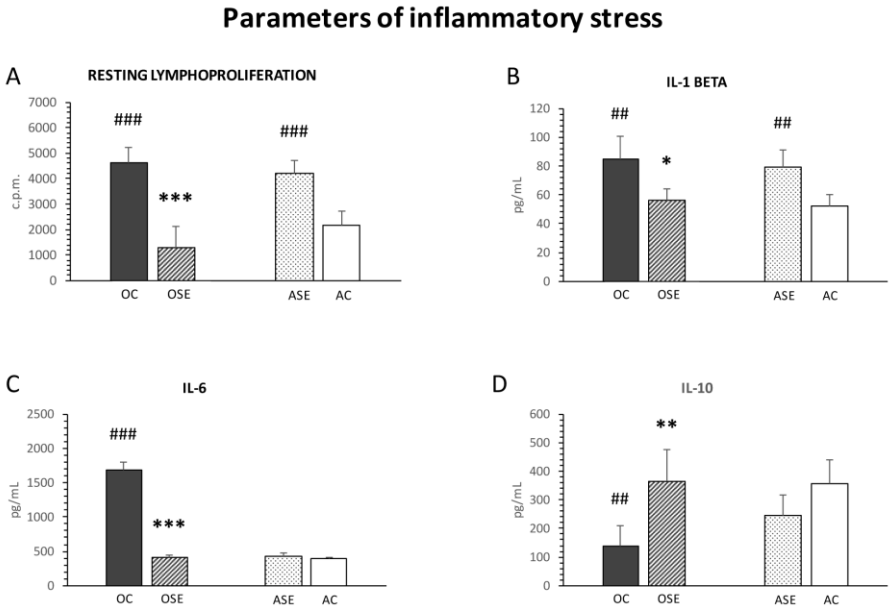

Figure 4