

Age-Related Cognitive Decline in Female C57BL/6_{cep} Mice 15-16 Months of Age

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Abstract

Understanding cognitive aging is becoming more relevant as the elderly population grows. Age correlates strongly with a variety of adverse health outcomes. Rodent models of C57BL/6_{cep} strain have been used successfully to study the behavioral and neurophysiological changes associated with cognitive aging, but the majority of studies have focused on males. The aim of this study was the characterization of aged female C57BL/6_{cep} mice of 15-16 months of age as a model of aged-related cognitive decline (ACD) from the evaluation of behavioral, pathological and biochemical markers. Twenty young and 20 aged C57BL/6_{cep} female mice were used. Aged C57BL/6_{cep} mice showed cognitive impairment related with age, according to the evaluation of different mechanisms of memory. In the case of the hot plate test to assess pain sensitivity (nociception) based on the effect of heat, it was found that aged animals responded significantly later to heat than young animals. It was observed an increase in glucose concentration and in the population of nuclear polymorphs in serum. On the contrary, the population of lymphocytes was significantly lower in the aged mice in relation to the young animals. At the age of 15-16 months, a significant decrease in the size of the thymus and brain was obtained. The correlation of results obtained in relation of behavioral task, hematological, biochemical and histological analyses indicates that they are a useful model of ACD, and that it can be used to characterize this disorder and evaluate drugs for its treatment.

Keywords: Aging; Mice; Cognitive Decline; Learning; Memory; Behavioral Tasks

List of abbreviations: AD: Alzheimer's disease; ACD: Age-related cognitive decline; MWM: Morris Water Maze; IC: Illuminated chamber; DC: Dark chamber; EDTA: Ethylenediaminetetraacetic acid; NPM: Nuclear polymorphs; SD: Standard deviation

Introduction

Age correlates strongly with a variety of adverse health outcomes. Examples include cancer, heart disease, arthritis, and cognitive decline. The terms “aging” and “senescence”, although used interchangeably, differ. Aging, in a broad sense, includes all of the biological changes that occur over the course of a lifetime, from the developmental milestones of infancy, childhood, and adolescence to the changes that occur in the later stages of life. Senescence refers to normal impairments that are characteristic of older ages, which are progressive and time-dependent, such as muscle atrophy, menopause, loss of hearing and/or vision, grey hair, and loss of skin elasticity [1].

Understanding the neurobiological effects of aging is becoming increasingly critical since the average age of the human population is increasing. Age-related cognitive decline (ACD) is a particularly insidious problem that can drastically affect quality of life independent of overt physiological disease [2]. ACD is associated with declines in spatial and non-spatial learning and memory, and with marked impairments in executive functions such as working-memory and attention [3]. Studies of brain morphology and function in ACD have revealed declines in regional brain volume and cortical activation in the pre-frontal cortex and medial-temporal lobe, including the hippocampus [4].

Rodent models of C57BL/6 strain have been used successfully to study the behavioral and neurophysiological changes associated with cognitive aging, but the majority of studies have focused on males. In addition, a few studies have compared the effects of aging on cognition between male and female rats, and have suggested that females decline in cognitive function sooner than males [5-6]. These observations, combined with the fact that women possess a longer average lifespan, underscore the importance of characterize an ACD's model in female mice.

The study of animal models has played an important role in progress toward understanding of the neurological/neurochemical alterations underlying ACD and has led to identification of many potential therapeutic interventions. However, the development of additional interventions, particularly those targeted at prevention or arrest of neurological decline, may require a more complete understanding of the time-related biological processes which lead to the neurological and immune alterations responsible for cognitive dysfunction [7]. Much of the reported studies on ACD in mice is focused on biogenic animals with Swedish APP and A246E PS1 mutations and not in mice where aging occurs naturally [8]. Taking into account the age relationship between mice and humans, mice should be at least 10 months old for in-

clusion in a middle age group which is the reported age at which the manifestations of the ACD in mice occur. The upper limit to consider an aged mouse is about 14–15 months, at which point most biomarkers are changing [1].

For all these reasons, it is necessary to characterize a natural aged mice model of cognitive decline associated with age and to be able to explain by other biomarkers how they are related to this pathology. The aim of this study was the characterization of aged female C57BL/6cnp mice of 15-16 months of age as a model of ACD from the evaluation of behavioral, pathological and biochemical markers.

Materials and methods

Animals and housing conditions

Young (3-4 months, n=20) and aged (15-16 months, n=20), were purchased from Centro Nacional para la Producción de Animales de Laboratorio (CENPALAB, La Habana, Cuba). All mice were given space with a controlled consistent temperature (21 ± 3 °C) and lighting environment (12 h/12 h light/dark cycle). Mice were fed with the EAO1004_{cnp} diet for rodents and water *ad libitum*. The animals were adapted for a week to the experimental conditions prior to the beginning of the experiments and then mice were individual identified by ears perforation nomenclature. Animals were placed in polypropylene cages with wood chip bedding (Sournid, Spain), at the rate of 10 animals per cage. All experimental protocols related to the use of animals were approved by the Institutional Animal Care and Use Committee at Centro Nacional de Biopreparados (BioCen, Mayabeque, Cuba).

Experimental design

Body weight was measured, and cognitive function was evaluated by the application of different behavioral tests.

Behavioral tasks

The animals were transferred to the room where the behavioral tests were carried out 24 h before the beginning of the assays. At the end of each task, the surfaces of the mazes were disinfected with 70% ethanol solution.

Y-maze task (spontaneous alternation): allows evaluating the spatial memory of short-term work [9] and in this study it was used the methodology described by García and Esquivel (2018) [10]. In the spontaneous alternation task, it was considered that the mice made alternations when they sequentially visited the three arms, without repeating any. We also counted

the number of repeated entries to the same arm (perseveration) and incorrect entries to the arms for each animal. Finally, spontaneous alternation was calculated by the following expression:

$$\text{Spontaneous alternation (\%)} = \frac{\text{Alternations}}{\text{Total of possible alternations}} \times 100$$

$$\text{Total of possible alternations} = \text{Total of entries} - 2.$$

Morris water maze (MWM): evaluates spatial learning and memory in mice and is one of the most frequently used laboratory tools in behavioral neuroscience, originally described by Morris, 1984 [11-12]. The device consists of a large circular pool filled with water of 120 cm in diameter, inside which a plastic platform was placed, the upper base of which is 6x6 cm that had to be located by the animal. The water temperature was kept between 26 and 27 °C.

During 3 days of training, animals learn to find the platform and escape from the pool, the platform was kept in a fixed location and the animal was placed in a different position each day according to the points marked on the pool wall. Four daily training were carried out and at the beginning of each training, animals were placed with its back to the platform. Time in which animals found the platform in each daily training was recorded to make a learning curve. In each training section animals swam for 90 s until they found the platform. If the animal passed this time without finding the platform, they were helped to find it. Animals being allowed to remain on the platform for a period between 10 and 30 s. The retention of the test was measured on the 4th day without a platform; all the animals were placed in the same position and swam for 60 s.

The animal's behavior was recorded with a non-professional digital camera and was evaluated by a trained specialist in the computer with the stopwatch tool of window. The analysis of the videos accounted for the time each animal swam in each quadrant and the time it took to go to quadrant I for the first time, where the platform was located. This results were normalize by latency differences on the first few trials (before the young mice have learned) to examine the change in latency over time within an age group.

Passive avoidance test: is based on the innate ability of rodents to recall aversive stimuli and avoid them [12]. In this study it was used the methodology described by García and Esquivel (2018) [10]. It was used a box with an illuminated chamber (IC) connected to dark chamber (DC) by a guillotine door. In the DC, there is a closed circuit that produces small electric discharges. The task included a first phase (habituation) where the

mouse was placed in IC with the guillotine door closed. After 25 s, the guillotine door was opened and the mouse was expected to spontaneously go to it. Mice that in this part of the task did not go to the DC during 180 s or more were rejected. When the mouse spontaneously entered with all four legs in the DC, the guillotine door was closed and it received an electric shock of 0.3 mA during a time of 5 s. The animals were kept inside the DC for 10 s after receiving the electric shock (retention). After 24 h mice came back to the passive avoidance task with free access to both chambers for 300 s. The time that mice delay in entering for the first time into the DC was recorder and considered as latency transition. The escape latency data were normalize by latency differences on the first few trials (before the young mice have learned) to examine the change in latency over time within an age group.

Hematological and biochemical determinations

Ten aged animals and 10 control animals from 3-4 months of age were used. The animals were anesthetized in a ketamine and xylazine mix and approximately 100 µL of blood was extracted by the retro-orbital plexus, which was added the ethylenediaminetetraacetic acid (EDTA) anticoagulant solution. This sample was analyzed in the optical microscope (Olympus, Tokyo, Japan), with the objective of 100 × immersions, and 100 cells were observed to calculate the percentages corresponding to the nuclear polymorphs (NPM) and the lymphocytes. The rest of the blood was then collected to obtain the serum. In the serum sample collected, the concentration of glucose was measured with the Rapi-Glucotest reagent kit.

Histological studies

Histological analyzes were performed in 10 young animals and 10 aged animals. The animals were sacrificed by cervical dislocation and the brain, thymus, spleen, liver, kidneys and lungs were collected. Organs were rinsed with cold 0.9% NaCl to eliminate rest of blood, dried with filter paper and were weighted using an analytic balance (Sartorius, Göttingen, Germany). The relative weight of the organ was calculated in relation to the body weight of the animal before sacrifice. All mice organs, except the thymus, were fixed in phosphate buffered 10% formalin solution. After inclusion in paraffin, the organs were cut and stained with hematoxylin/eosin according to conventional techniques. The histological evaluation of organs and tissues was carried out with an optical microscope (Meiji Techno, Tokyo, Japan).

All reagents used in sections 2.4 and 2.5 were supplied by Sigma-Aldrich, Merck, Darmstadt, Germany, except the Rapi-Glucotest reagent kit, which was supplied by HELFA® Diagnostics, Havana, Cuba.

Statistical analysis

Student t test was used to identify significant differences between experimental groups (GraphPad Prism 5 for Windows, Release 5.03, Standard Version, San Diego, CA, USA). All values were expressed as the means \pm standard deviation (SD) and a P value <0.05 was considered significant.

Results

Body weight

The body weight of female C57BL/6_{cep} mice at the age of 15-16 months was significantly greater than the control group from 3-4 months (Figure 1).

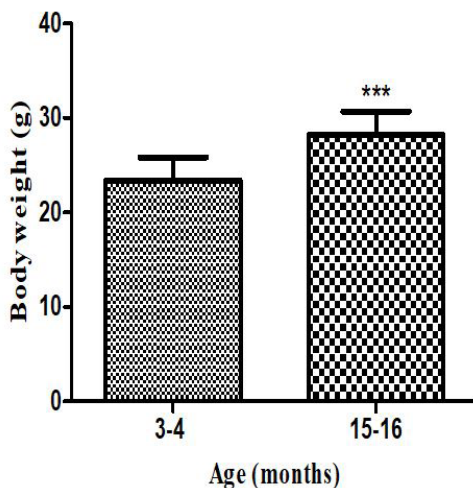


Figure 1: Body weight of female C57BL/6_{cep} mice up to 15-16 months of age. Data (n=10) shown are the means \pm SD. * indicates $P < 0.05$ compared to young control group (3-4 months of age)

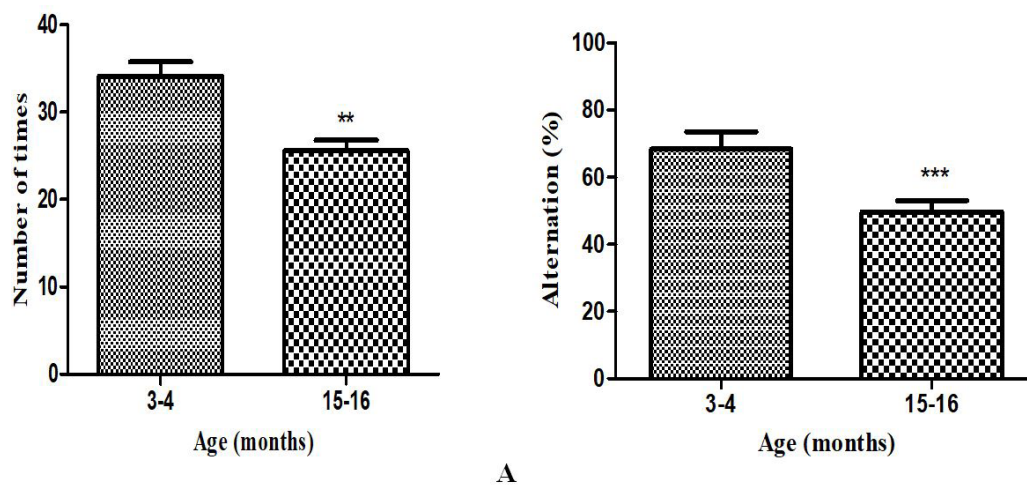


Figure 2: Y maze task of female C57BL/6_{cep} mice up to 15-16 months of age. A) Number of entries to each maze's arm. B) Percentage of alternation. Data (n=10) shown are the means \pm SD. * indicates $P < 0.05$ compared to young control group (3-4 months of age)

Behavioral tasks

A significant difference in relation to the number of entries obtained in both experimental groups in the Y maze test was observed (Figure 2A). The percentage of alternation was significantly lower in animals from 15-16 months of age than in the young animals from 3-4 months of age (Figure 2B).

The second behavioral task used to evaluate if the naturally aged C57BL/6_{cep} mice had cognitive impairment was the MWM. All mice swam normally with the usual adult swimming posture and had no difficulty climbing onto the platform. Overall analysis of escape latency during training (Figure 3A) showed a clear difference between both groups of animals, being aged animals more slowly to find the platform. During the three days training, a decline in escape latency was obtained for both young and aged animals. During the retention phase of the test, it was observed that the young animals of 3-4 months of age were swimming for a significantly longer time in the platform quadrant (Figure 3B) than in the rest of the quadrants, which didn't occur with the aged animals. It was also achieved that young animals found the quadrant where the platform was located during the acquisition phase significantly faster than the aged ones (Figure 3C).

The other behavioral task used was the passive avoidance test (Figure 4). In this task the young animals were performed significantly better than aged animals because their latency in entering the DC was lesser.

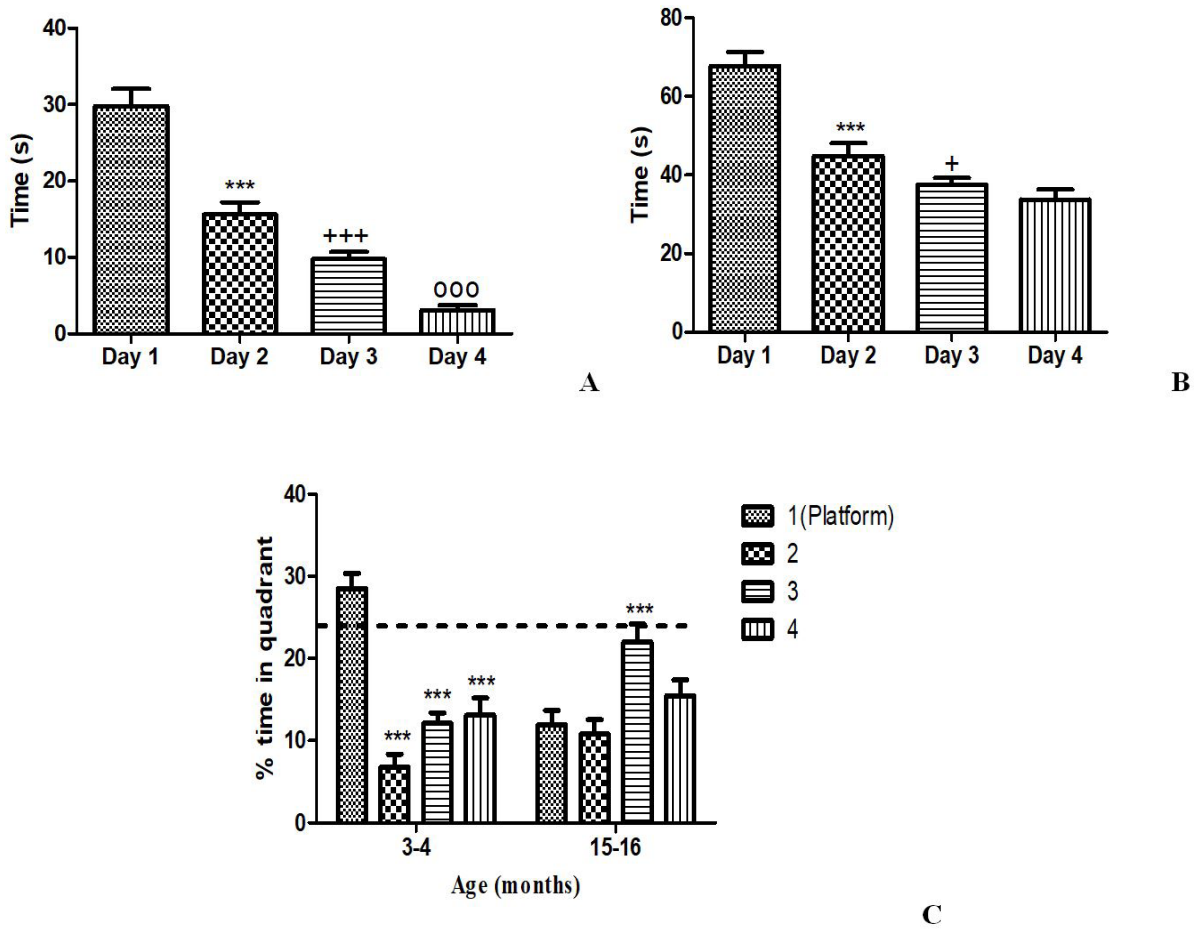


Figure 3: Morris water maze task of female C57BL/6_{cenp} mice up to 15-16 months of age. A) Change in latency over time in young animals. B) Change in latency over time in aged animals. C) Percentage of time in each quadrant. Data (n=10) shown are the means±SD. * indicates $P < 0.05$ compared to young control group (3-4 months of age). + indicates $P < 0.05$ compared to day 2 of training. o indicates $P < 0.05$ compared to day 3 of training

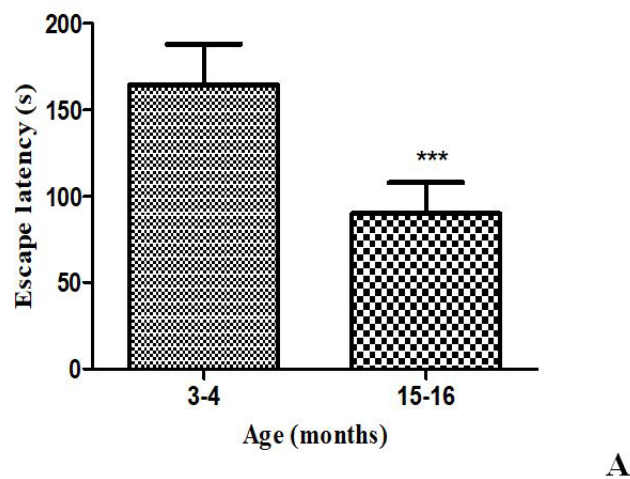


Figure 4: Escape latency in female C57BL/6_{cenp} mice up to 15-16 months of age. (A) Passive avoidance test. Data (n=10) shown are the means±SD. * indicates $P < 0.05$ compared to young control group (3-4 months of age)

Hematological and biochemical determinations

One of the hematological parameters examined was the population of lymphocytes which in the group of 15-16 month of age decreased significantly in relation to young animals (Figure 5A). On the other hand, the population of NPM (Figure 5B) in animals 15-16 months of age was significantly higher than in animals from 3-4 months of age. The concentration of glucose in the

serum showed an increase in the group of 15-16 month of age in comparison with the animals of 3-4 months of age (Figure 5C).

It was found that the weight of brain and thymus in mice of 15-16 months of age was significantly lower than in the control mice group (Figure 6A-6B). As a result of the comparison between the groups for weight of spleen, lungs and liver, was observed a significant increase in the aged animals of 15-16 months in relation to control group (Figures 6C-6E).

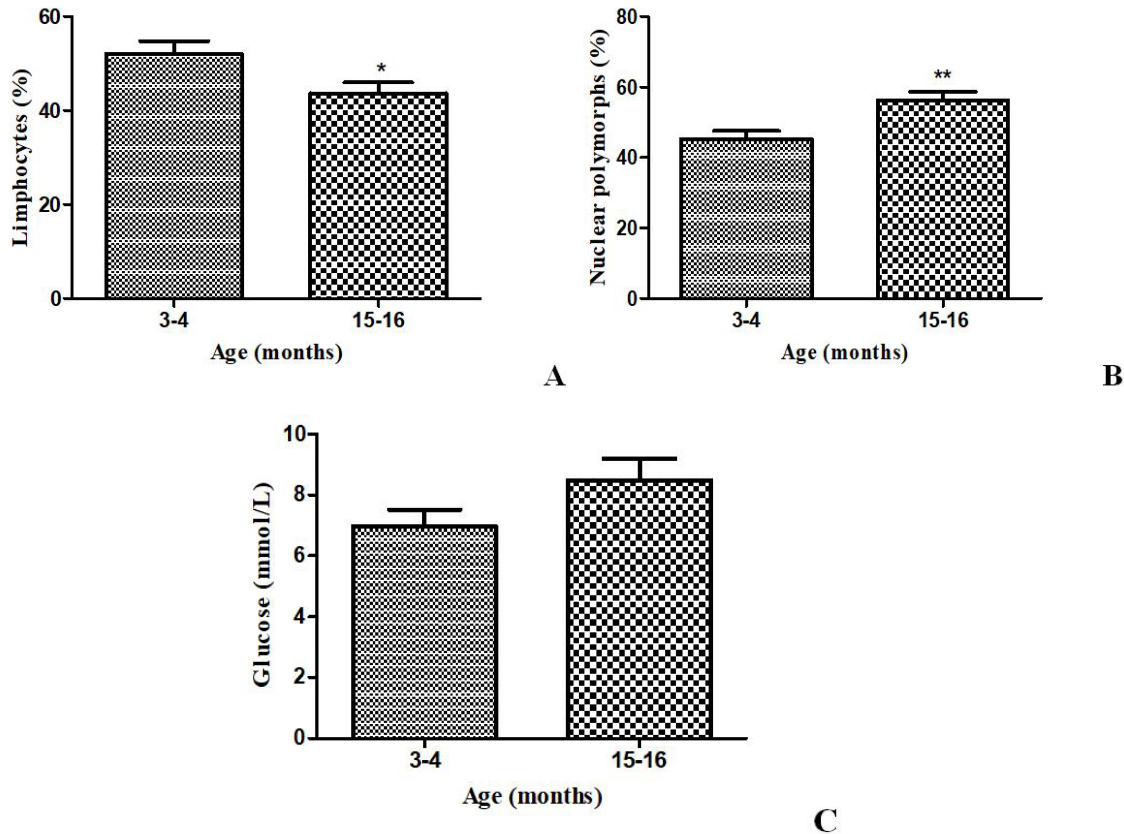
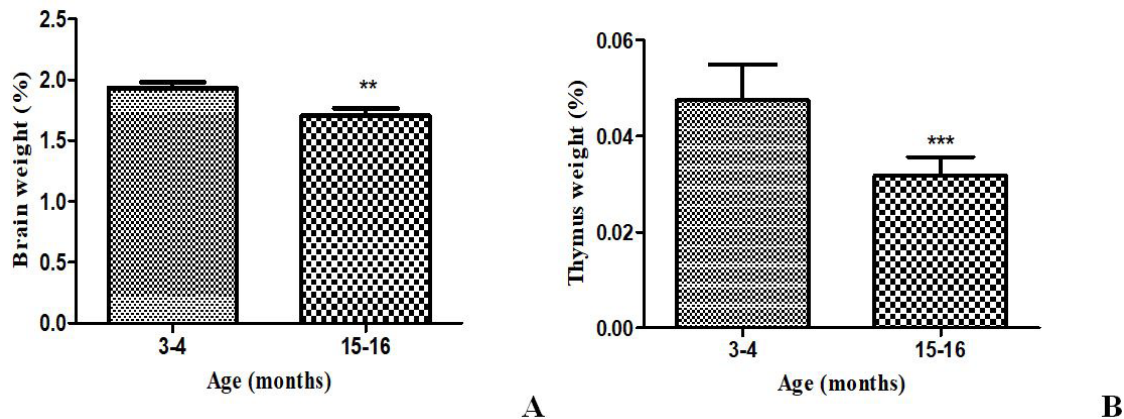


Figure 5: Hematological parameters and glucose concentration of female C57BL/6_{cep} mice up to 15-16 months of age. (A) Lymphocytes. (B) Nuclear polymorphs. (C) Glucose. Data (n=10) shown are the means±SD. * indicates P < 0.05 compared to young control group (4-5 months of age)



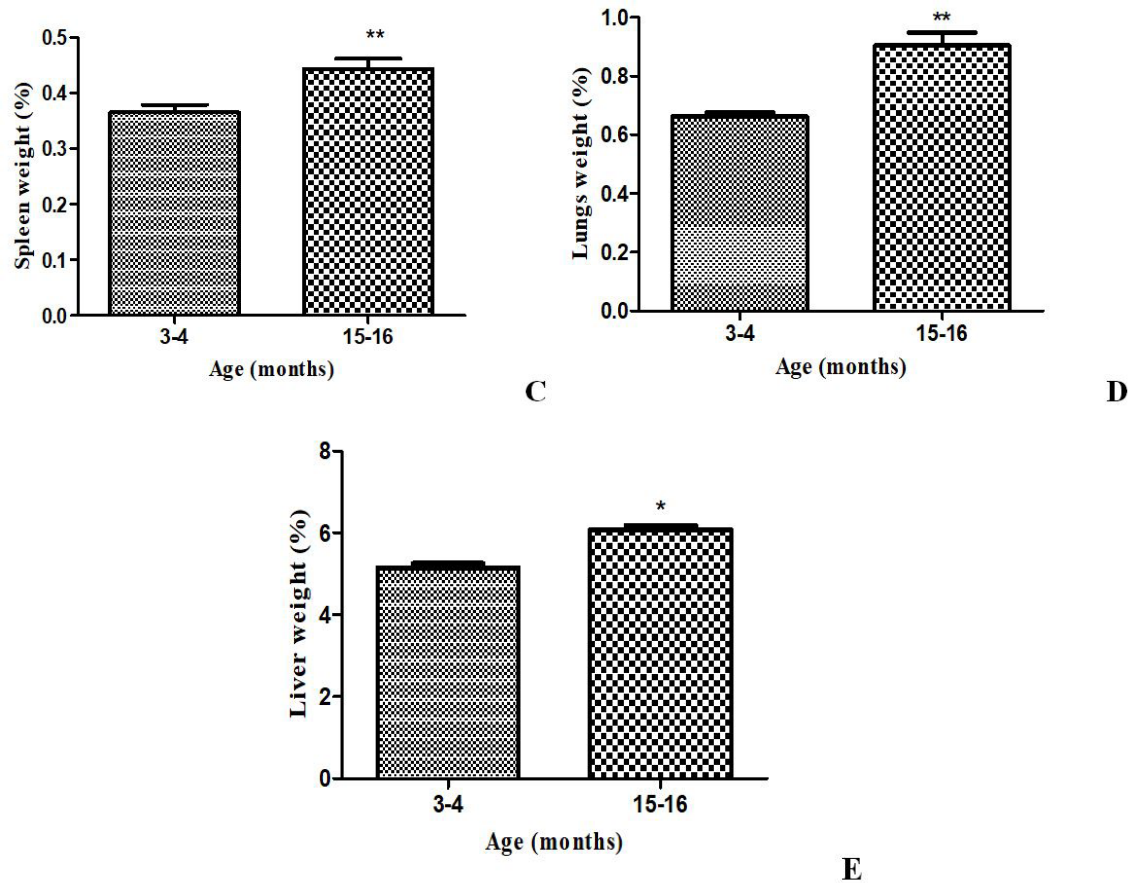


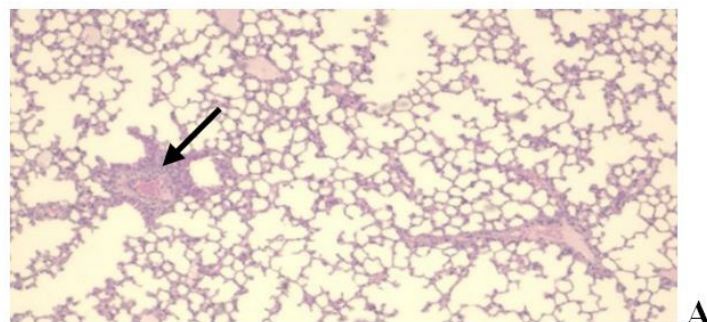
Figure 6: Weight of the organs relative to the body weight of female C57BL/6_{cenp} mice up to 15-16 months of age. (A) Brain weight. (B) Thymus weight. (C) Spleen weight. (D) Lungs weight. (E) Liver weight. Data shown are the means±SD. * indicates $P < 0.05$ compared to young control group (3-4 months of age)

Histological studies

Among the histological studies, the morphology of the lung was evaluated and was observed a minimal infiltration of lymphocytes and few macrophages associated with blood vessels in 20% of the animals in the young control group. In addition, in 70% of the aged animals, thickening of the alveolar septa and some areas with lymphocytic infiltration were observed in the lung, that did not predominate in the entire organ since most of the regions presented without any affectation (Figure 7).

No alteration was observed in the liver of young animals (Figure 8A). In the case of aged animals, little perivascular lymphocytic infiltration was observed in 20% of them in some regions (Figure 8B). Focal lipidosis was also observed in 40% of aged animals (Figure 8C), which appears to be associated with liver aging.

On the other hand, the morphological characteristics of the cerebellum (Figure 9) were normal for both young control animals and those aged 15-16 months of age.



A

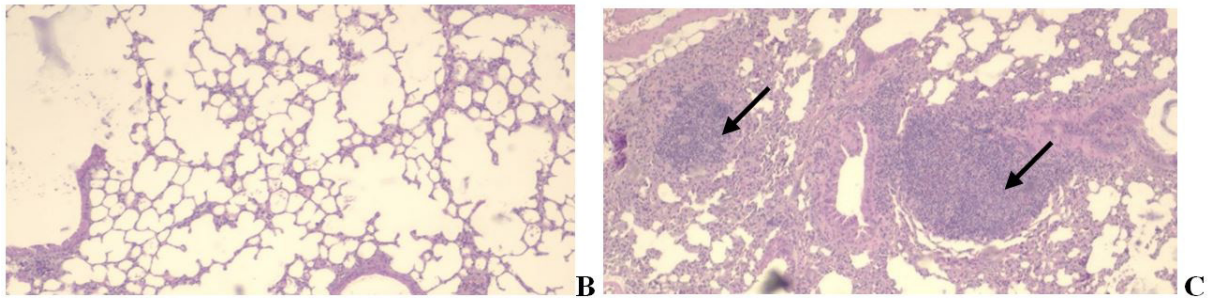


Figure 7: Damages presented in lungs of female C57BL/6_{cep} mice up to 15-16 months of age. (A) Minimal infiltration of lymphocytes in lungs of young control group (3-4 months of age). (B) Non-affected regions in lungs of aged mice (C) Thickening of the alveolar septa and lymphocytic infiltration in aged mice. HE 40X

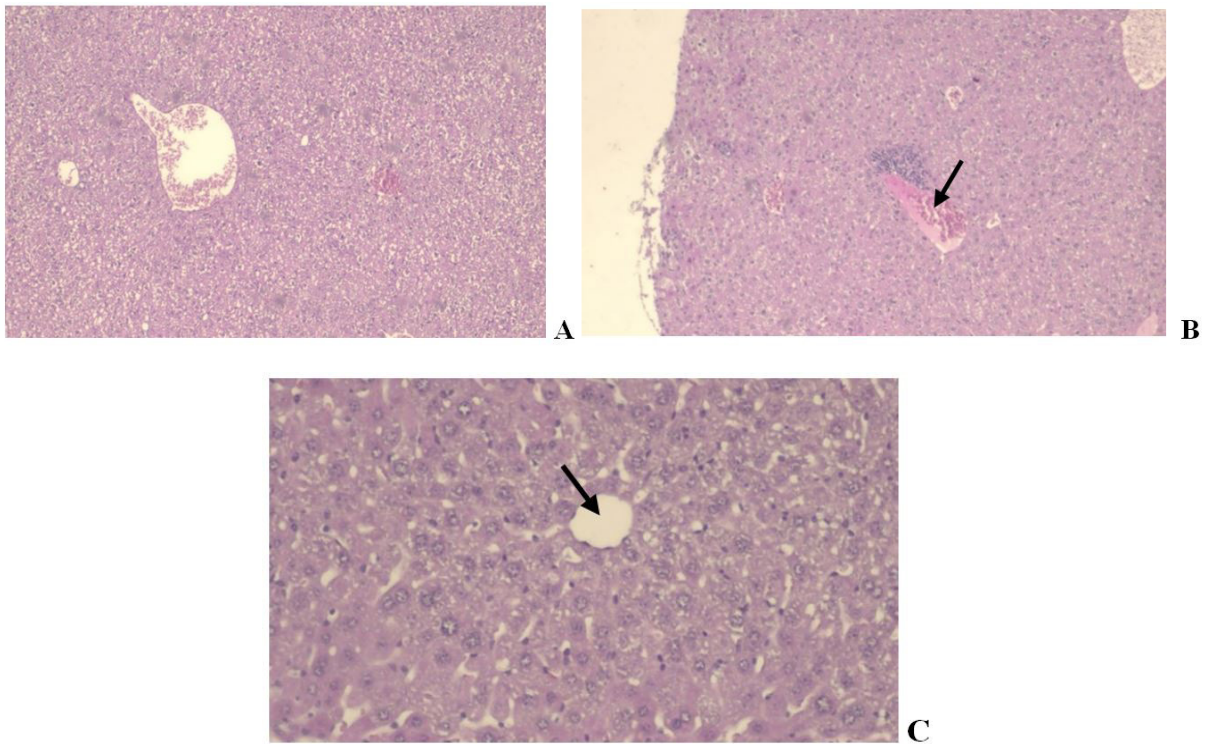


Figure 8: Damages presented in liver of female C57BL/6_{cep} mice up to 15-16 months of age. (A) Normal morphology of the liver of young control animals. HE 40X. (B) Perivascular lymphocytic infiltration in aged mice. HE 40X. (C) Macro focal and micro vesicular lipodosis in aged mice. HE 40X

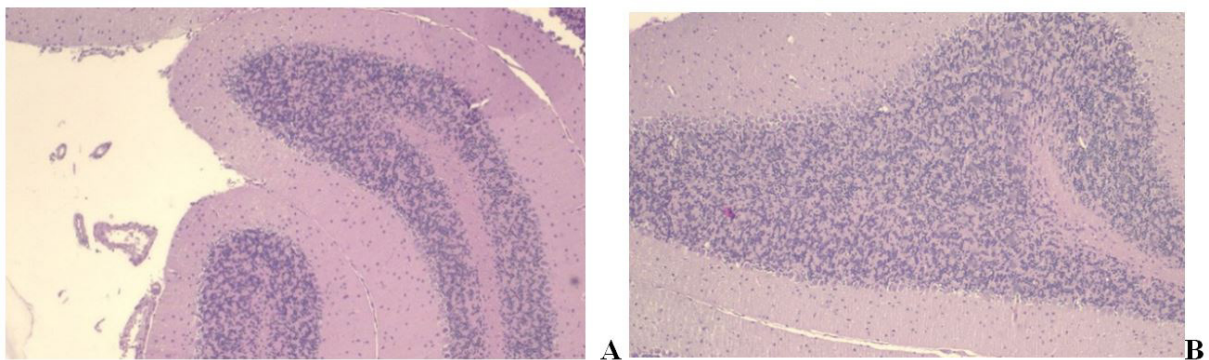


Figure 9: Normal brain of female C57BL/6_{cep} mice up to 15-16 months of age. (A) Cerebellum of mice from the young, healthy control group. (B) Cerebellum of the aged, healthy group. HE40X

Discussion

In accordance with several studies, an increase in mice body weight until 15-16 months of age was observed (Figure 1) [14-16]. These results indicate that the animals were healthy.

The results obtained in the Y-maze test (Figure 2A, 2B) suggested that aged animals showed a decrease in the ability to orient themselves in the space and they didn't show the natural instinct to explore new places that have been reported for young male mice of 9 weeks of age without being subjected to any drug or treatment [17, 18].

The results observed in the MWM (Figure 3) demonstrated the cognitive decline in the group of aged animals regarding the latency period during the 3 days of the acquisition phase (Figure 3B), since these animals delayed significantly the time to visit the platform quadrant for the first time. The fact that aged animals had the worst performance in relation to this variable in comparison with young animals (Fig. 3A) has been described in the literature and R. D'Hooge and P.P. De Deyn (2001) [19] stated that when animals age they show a decline in cognitive functions and have a decrease in the efficiency of the execution of this behavioral test. In the aged animals the abilities to swim, locomotion and exploration change. It has been argued that this has coincided with structural and physiological modifications in some brain structures, specifically in the hippocampus, related to spatial learning. As a result of the analysis of the residence time in each quadrant (Figure 3B) in the second phase of the test, the presence of cognitive deterioration was confirmed in the aged mice. The greater permanence in the platform quadrant by the animals of the young control group shows that these animals performed better during the retention phase. This is a spatial preference test in which if the animal has learned in the final phase of the test, it will swim longer in the target quadrant, that is, where the platform was previously located because the associated memory mechanisms will have been correctly activated withholding [19].

The passive avoidance test (Figure 4A), meanwhile, is traditionally used as a quick and easy way to explore short and long-term memory. In this case, it was used to measure long-term memory and it was found that in aged animals there was cognitive deterioration since it took less time to return to the DC where they received the aversive stimulus. This test induces in the rodent a conflict between the innate preference for dark areas as it is a kind of nocturnal habits, and the aversion for bright areas. It is a one-way test that allows you to study acquired learning and memory. The animal is conditioned with an aversive stimulus and is subsequently evaluated if it remembers that experience [18].

Therefore, based on the different mechanisms of memory that is evaluated in the Y-maze test, in the MWM and in the passive avoidance test, it can be affirmed that aged female C57BL/6cnp mice of 15-16 months show cognitive impairment related with age.

Regarding the increase in glucose concentration (Figure 5C) that was obtained in aged animals, it has been shown that the lowest brain metabolism of glucose in humans is present before the start of a clinically measurable cognitive impairment in groups of people with risk of Alzheimer's disease (AD) [21]. Furthermore, in vitro and animal studies have shown that cerebral hypometabolism can precede and contribute to the neuro-pathological cascade that causes cognitive decline in AD. On the other hand, this hypometabolism of glucose in the brain may be due to defects in glucose transport in the blood-brain barrier, in glycolysis and / or in mitochondrial function [21], and therefore this physiological situation leads to elevated serum levels of this molecule during aging.

According with the reported by Shaw *et al.*, (2010) [22] the balance between the generation of myeloid lineages (monocytes and granular leukocytes, such as NPM, neutrophils, basophils and eosinophils) and lymphoid (T, B and NK lymphocytes), characteristic of young individuals, is lost with aging [Figures 5A, 5B], showing a tendency towards myeloid progenitors to the detriment of lymphoid. In the case of the lymphoid fraction of the animals of 15-16 months of age (Figure 5A), it supposes that these decreases are related to the thymus involution that occurs during aging, and in particular, the progressive loss of the thymic epithelial space, where the thymopoiesis takes place [22].

In agreement with studies in BALB/c aged mice, our data reveal a strong increase in spleen weight (Figure 6C) at the age of 15-16 months [23]. Some studies have shown age-related white pulp atrophy in the spleen of Sprague-Dawley rats which correlate with a decrease of greater than 80% in lymphocyte number with aging [24]. The results obtained in this work with relation to weight of thymus and spleen (Figures 6B, 6C) may explain the decrease in the lymphocyte population obtained in aged mice (Figure 5A) because in these organs take place lymphocyte production.

In the case of the finding of the decrease in brain size in mice aged 15-16 months (Figure 6A), this result agrees with that reported by Esquivel *et al.*, (2020) [23] in an aged BALB/cnp mouse model. In addition, it has been found post-mortem that the size of the brains of people with AD is lower than that of healthy people of the same age [25]. The decrease in brain

weight is associated with the neuronal death described in AD [26], therefore this result contributes to collect experimental evidence to use aged C57BL/6cnp mice as model of ACD.

The significant increase in the weight of the lungs (Figure 6D) obtained in aged animals to 15-16 months corresponds to that reported in the literature and may be related to lung diseases associated with dementia [24]. Furthermore, this result may be related to the lesions observed in lung histology in aged mice of 15-16 months of age (Figure 7C). Many studies have showed that cognitive function is impaired in patients with lung disease with or without hypoxemia. Cerebral disturbance is noted in patients with lung disease and may be related to hypoxia in the brain [27].

Moreover, the focal lipidosis in liver obtained in C57BL/6cnp mice at 15-16 month of age (Figure 8C) suggest the presence of an increase of circulating lipids. In the liver, aging causes an increase in lipid accumulation (micro and macro vesicular lipidosis) as a result of multiple alterations in lipid metabolism, among which are a lower β -oxidation and/or an increase in de novo synthesis [28]. Therefore, steatosis found in pathological studies is likely to be associated with the renal aging process and not with AD.

The animal models evaluated for ACD generally use the male sex, when it is in women that the highest incidence and severity of this disorder has been reported, generally leading to AD [6]. It has been reported in part that female mice due to hormonal changes aren't an effective model for this pathology [7]. However, in this experiment it has been shown that female C57BL/6cnp mice respond to applied behavioral tests and have biochemical and histological characteristics that are present in this pathology. On the other hand, the use of mice of this strain constitutes an advantage over the BALB/c strain in which difficulties in its management have been reported for the male sex due to the appearance of frequent fights in the housing cages. This situation prevents the normal development of the experiment, which led to the need to house the animals individually [23, 29]. Individual housing isn't convenient since they are gregarious animals and neurologically mice can be affected. The literature recommends the search for solutions to modulate aggression among male mice housed in groups with environmental enrichment, using the female sex or a more tame strain before resorting to separating them [29].

Conclusions

Behavioral tasks, hematological, biochemical and histological tests results of aged C57BL/6cnp female mice indicate the usefulness of this rodent model for assessing ACD. We propose that this model can be used to characterize ACD and to evaluate drugs for its treatment. On the other hand, results provide experimental evidences of the female mice usefulness in the study of ACD, due to the highest incidence and severity of cognitive disorders among them.

Declarations

Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests to publish these results.

Funding

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Authors' contributions

Nashelly Esquivel Crespo: Conceptualization, Methodology, Software, Writing - Original Draft. **Yenela García Hernández:** Investigation, Validation, Writing - Review & Editing. **Mercedes Martínez Rabaza:** Data Curation. **Bestraida Lores Cintra:** Formal analysis. **Claudio Rodríguez Martínez:** Supervision, Project administration.

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