Characterization of Male OF1_{cenp} Mice with Age-Induced Cognitive Decline

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How to cite this paper: Nashelly Esquivel, Yenela García, Osmany Carvajal, Claudio Rodríguez. (2022) Characterization of Male OF1_{cenp} Mice with Age-Induced Cognitive Decline. *International Journal of Clinical and Experimental Medicine Research*, 6(2), 111-121.

DOI: 10.26855/ijcemr.2022.04.002

Received: December 12, 2021

Accepted: January 7, 2022

Published: February 14, 2022

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Abstract

Age is the greatest risk factor for memory loss and other disorders. The aim of this paper was to investigate whether the OF1_{cenp} mouse was suitable for use as a model of age-induced cognitive decline. To achieve this, it was compared male OF1_{cenp} middle aged and aged with young mice for learning abilities and biochemical markers. It was obtained that based on the different mechanisms of memory that was evaluated in the Y-maze test, in the Morris Water Maze and in the Novel Object Task, it can be affirmed that aged male OF1_{cenp} mice from 7-8 months show true cognitive deficits based in the reduction of learning and memory. It was found an increase in glucose, triglycerides and cholesterol concentration in the aged mice. At the age of 7-8 and 15-16 months, a significant decrease in the size of the brain was obtained, on the contrary the liver weight does not show differences. An increase in oxidative damage and a decrease in the antioxidant total capacity in the brains of aged animals was also found in relation to young animals. The correlation of results obtained in relation to behavioral task, hematological and biochemical analyses indicate that OF1_{cenp} mice with 15-16 months of age are a useful model cognitive decline. It can be used to characterize this disorder during aging and evaluate nutritional supplements for its prevention.

Keywords

Cognitive decline, Animal models, Aging, Mice, OF1

1. Introduction

Age-related cognitive decline (ACD) is a particularly insidious problem that can drastically affect quality of life independent of overt physiological disease. 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 [1].

Age is the greatest risk factor for memory loss and dementia which is defined as a cognitive decline that interferes with independent functioning [2]. Around 80% of dementia cases are represented by Alzheimer's disease worldwide and are characterized by different stages of cognitive and functional impairment [3]. Given the accelerated aging of the population, ACD and the diseases associated with it are becoming a growing health problem and, therefore, an urgent priority to be addressed as part of the lines of research aimed at achieving new pharmacological treatments [4].

Despite the spectacular advancement of techniques and knowledge in neuroscience, human research has significant limitations. These limitations make experimental studies with animal models of the different types of cognitive

dysfunction essential. However, no model is inherently good or bad, only that some have advantages over others according to the type of research that is carried out. Sex, species and genetic properties of the models must be carefully selected [5]. For example, some of these models of dementia have been induced by different substances (streptozotocin, scopolamine, and heavy metals) or have been obtained genetically. On the other hand, in animal models related to cognitive decline, dementia or Alzheimer's disease, naturally aged mice are also used where the symptoms of the disease appear coupled with the normal aging process. Two strains of mice commonly used during the preclinical phase of drug development for the treatment of memory loss or dementia are the inbred strains BALB/c and C57BL/6 [6]. Studies with these types of mice are generally not very variable and highly recommended when it comes to drugs. However, during the development of nutritional supplements with the aim of preventing cognitive decline or to improve the quality of life of elderly people, the use of non-consanguineous strains of mice may be useful to characterize a heterogeneous population.

Male OF1 mice have been characterized in the literature during aging-induced cognitive impairment, but taking into account only the behavioral part and without describing the biochemical or hematological changes that occur. Nor has it been taken into account in other studies what would be the minimum age at which these animals can be used to achieve greater efficiency in the studies, using the minimum number possible [7]. It is needful to determine the minimum age at which these animals show cognitive decline and which biochemical markers are modified. In the field of experimental neuropharmacology, it is required to use several animal models to determine the usefulness of a new formulation and its dosage, due to the complexity of cognitive function, in which multiple interconnected brain structures are involved [5].

As cognitive decline is not a homogeneous process, and because several types of memory processes exist involving different neural circuits, a set of behavioural tests was performed allowing measurement of age-related learning deficits in the OF1_{cenp} mice. The aim of this article was to investigate whether the OF1_{cenp} mouse was suitable for use as a model of age-induced cognitive decline. To achieve this, learning and memory abilities and biochemical markers of aged mice were compared with those of young mice.

2. Materials and Methods

2.1. Animals and housing conditions

Male OF1_{cenp} mice, young (2-3 months, n=20), middle aged (7-8 months n=20) and aged (15-16 months, n=20), were purchased from *Centro Nacional para la Producción de Animales de Laboratorio* (CENPALAB_(cenp), La Habana, Cuba) when they had reached the corresponding age. It was decided to use male mice since the females present different hormonal processes that can interfere with their performance in behavioral tests and make their handling difficult. They were kept in a conventional room with controlled environment (type IV barrier of minimum safety), with a temperature of $21 \pm 3^{\circ}\text{C}$ and relative humidity of 40 to 70%, effecting 18 changes of air per hour, 100% of external air injection with 85% filtration. Circadian rhythms were controlled using a 12h cycle light/darkness using an automatic timer. Mice were fed with the EAO1004_{cenp} 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 5 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).

2.2. Experimental design

Body and organs (brain and liver) weight was measured in each experimental time. 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 percentage in relation to the animal's body weight before sacrifice. Cognitive function was evaluated by the application of different behavioral tests. Also hematological markers in serum (cholesterol, triglycerides and glucose) and biochemical markers in brain homogenate (total antioxidant capacity and oxidative damage to lipids and proteins) were analyzed in each experimental group.

2.3. Behavioral tasks

The animals were transferred to the place 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. Moreover, as it is difficult to separate the specific memory impairment from sensorimotor changes associated with aging, the use of various tests gave a better guarantee that results obtained represented true cognitive deficits.

Y-maze task (spontaneous alternation): it 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]. It was allowed that mice explore the Y-maze during 7 minutes. 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:

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

Total of possible alternations = Total of entries - 2.

Novel object recognition task (NOR): it is based on the natural instinct of rodents to explore novel objects and evaluate non-spatial memory [11]. The task was performed under the conditions described by García and Esquivel (2018) [10].

The percentage of time that mice spent recognizing the Novel object (NO) and Familiar Object (FO), relative to the total object recognition time or Preference index (PI), was calculated by the following expressions:

$$\text{IP NO (\%)} = \frac{\text{Time Exp. NO}}{\text{Time Exp. FO} + \text{Time Exp. NO}} \times 100$$

$$\text{IP FO (\%)} = \frac{\text{Time Exp. FO}}{\text{Time Exp. FO} + \text{Time Exp. NO}} \times 100$$

Time Exp. FO: Time of FO exploration

Time Exp. NO: Time of NO exploration

Morris water maze (MWM): it was used to evaluate spatial learning and memory in mice and is one of the most frequently used laboratory tools in behavioral neuroscience. The task was performed under the conditions described by Esquivel et al. (2021) [1].

In each training session, the animal swam for 90s until it found the platform. If the animal passed this time without finding the platform, it was helped to find it. The animal was allowed to remain on the platform for a period between 10 and 30s. 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 60s.

The animal's behavior was recorded with a non-professional digital camera and was analyzed by a trained experimenter 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 1 for the first time, where the platform was located (Escape latency).

2.4. Hematological and Biochemical determinations

Ten animals of each experimental group 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 to obtain the serum. In the serum sample collected, the concentration of glucose, triglycerides and cholesterol was measured with the Rapi-Glucotest, Monotriglitest and Colestest reagent kit respectively.

The animals were sacrificed by cervical dislocation and brain was collected to obtain brain homogenates in Tris buffer at pH 7.6 containing 0.32 M sucrose, 10 mM Tris and 1 mM Ethylenediaminetetraacetic acid and differential centrifugation was performed to obtain the post-mitochondrial fraction, according to the method described by Uwe and Von Hagen (2009) [12]. The supernatant was dispensed in aliquots of 1 mL, which were stored at -80°C.

In these homogenates the oxidative damage to lipids and proteins was measured. The oxidative damage to the lipids was measured from the thiobarbituric acid reactive substances (TBARS) method according to Okawa et al. (1979) [13]. The TBARS were determined from the molar extinction coefficient of the malondialdehyde ($\xi = 155 \text{ mM}^{-1}\text{cm}^{-1}$). The concentration of proteins in the tissue homogenate was determined by the modified Lowry method for biological tissues [14]. In addition, to quantify the oxidative damage to proteins, it was used the method described by Resnick and Parker (1994) [15].

Also in the brain homogenate the total antioxidant capacity was determined according to the instructions of the TAS reagent kit.

All reagents used in section 2.4 were supplied by Sigma-Aldrich, Merck, Darmstadt, Germany, except the Rapi-Glucotest, Monotriglitest and Colestest reagent kits, which were supplied by HELFA® Diagnostics, Havana, Cuba and TAS reagent kit which was supplied by RANDOX, England.

2.5. Statistical analysis

GraphPad Prism software 5.0 (United State) was used for all statistical analysis. One-way ANOVA and the post hoc Tukey's multiple comparisons test were used for analysis between the mice groups. All values were expressed as the means $\pm$ standard deviation (SD) and a P value <0.05 was considered significant.

3. Results

3.1. Body weight

The body weight of male OF1_{cenp} mice showed statistically significant differences from the age of 7-8 months, whose weight was significantly greater than the control group of 2-3 months (Figure 1). Among the aged mice groups, there were no statistically significant differences.

Figure 1. Body weight of male OF1_{cenp} mice up to 15-16 months of age. Data shown are the means $\pm$ SD. * indicates $P < 0.05$ compared to young control group (2-3 months of age).

3.2. Behavioral tasks

A significant difference in relation to the number of entries obtained in both aged experimental groups in relation with the young control group in the Y maze test was observed (Figure 2A). The percentage of alternation was significantly lower in animals of 7-8 and 15-16 months of age than in the young animals of 2-3 months of age (Figure 2B). Among the both aged mice groups there were no statistically significant differences in any case.

Figure 2. Y maze task of male OF1_{cenp} mice up to 15-16 months of age. (A) Number of entries to each maze's arm. (B) Percentage of alternation. Data (n=20) shown are the means $\pm$ SD. * indicates $P < 0.05$ compared to young control group (2-3 months of age).

The second behavioral task used to evaluate if the naturally aged $OF1_{cnp}$ mice had cognitive impairment was the NOR. In this task (Figure 3), there were obtained significant differences between the PI for FO and NO in animals of 2-3 and 7-8 month of age. In the case of the mice of 15-16 months there were no significant differences in relation with the PI. In the young control the PI for NO was significantly higher than for FO, opposite to occurred with middle aged animals (7-8 months).

The other behavioral task used to evaluate if the naturally aged $OF1_{cnp}$ mice had cognitive impairment was the MWM. This task is used to assess spatial learning, a special type of learning sensitive to hippocampal damage, an area affected by aging.

All mice swam normally with the usual adult swimming posture and had no difficulty climbing onto the platform. The latency to finding the hidden platform decreased significantly over the successive learning trials in three groups. During the retention phase of the test, it was observed that young animals of 2-3 months of age were swimming for a significantly longer time in the platform quadrant (Figure 4A) than in the rest of the quadrants, which did not occur with the aged animals of 7-8 and 15-16 months. It was also achieved that young animals found the platform quadrant significantly faster than the aged ones of 15-16 (Figure 4B).

Figure 3. PI for FO and NO in the NOR of male $OF1_{cnp}$ mice up to 15-16 months of age. One-way ANOVA and Tukey's post-test were applied. Data (n=20) shown as means $\pm$ SD. * indicates $P < 0.05$ compared to FO in each group of animals.

Figure 4. MWM of male $OF1_{cnp}$ mice up to 15-16 months of age. (A) Percentage of time in each quadrant. (B) Escape latency in the retention phase of the test. Data (n=20) shown are the means $\pm$ SD. * indicates $P < 0.05$ compared to young control group (2-3 months of age).

3.3. Hematological and biochemical determinations

While the brain weight decreased significantly as the age of the animals advanced, the liver weight didn't show significant differences (Figure 5).

The concentration of glucose (Figure 6A), triglycerides (Figure 6B) and cholesterol (Figure 6C) in the serum showed a significant increase in the aged group of 15-16 months in relation with the young animals of 2-3 months of age. In the case of the middle aged group of 7-8 months of age, significant differences only were found in the case of triglyceride concentration in relation to young animals.

In the brain homogenate it was shown that oxidative damage to lipids was significantly lower than in the young control group than in both groups of aged animals (Figure 7A). On the other hand, oxidative damage to proteins showed a significant increase in animals of 15-16 months compared to animals of 2-3 months of age but no in mice of 7-8 months (Figure 7B).

In the determination of the total antioxidant capacity (Figure 7C), the opposite was obtained.

Figure 5. Weight of the organs relative to the body weight of male OF1_{cenp} mice up to 15-16 months of age. (A) Brain weight. (B) Liver weight. Data (n=20) shown are the means $\pm$ SD. * indicates $P < 0.05$ compared to young control group (2-3 months of age).

Figure 6. Hematological parameters of male OF1_{cenp} mice up to 15-16 months of age. (A) Glucose. (B) Triglycerides. (C). Cholesterol. Data (n=20) shown are the means $\pm$ SD. * indicates $P < 0.05$ compared to young control group (2-3 months of age).

Figure 7. Biochemical parameters in brain homogenate of male $OF1_{cenp}$ mice up to 15-16 months of age. (A) Oxidative damage, TBARS as an indicator of damage to lipids. (B) Carbonylated proteins as an indicator of damage to proteins. (C) Total antioxidant capacity. Data ($n=20$) shown are the means $\pm$ SD. * indicates $P < 0.05$ compared to young control group (2-3 months of age).

4. Discussion

It is known that the course of aging and the diseases derived from it takes years, as well as all the behavioral, physiological and biochemical modifications that occur both in the aging of man and animals [4]. Therefore, the use of aged animal models in the development of therapeutic variants for cognitive decline constitutes an opportunity, which is very little exploited in the world.

In relation to the behavior observed in body weight, in accordance with several studies, an increase in mice body weight until 8-9 months of age was observed and after this experimental time body weight then remains constant (Figure 1) [1, 16-18]. Therefore, the results obtained show that the housing and feeding conditions used were adequate.

Regarding the results of behavioral tasks, these showed that as age advanced, cognitive decline in animals was established (Figure 2-4). In the Y-maze of spontaneous alternation (Figure 2), it was obtained that aged animals show a decrease in the ability to orient themselves in space that is reported for young female and male mice of 2-3 months of age [1, 8]. The cognitive decline evidenced in this task as age advances may also be related to the fact that this unconditioned behavioral test causes anxiety in aged mice, which exceeds the rodents' natural instinct to explore new spaces. Mice are fearful and face the conflict of staying in the familiar, safer arms, or exploring a different arm that is potentially threatening [19].

The results obtained in the NOR (Figure 3) correspond to that reported by Navarrete et al. (2008) [5] who stated that in this test the expected result is that mice without cognitive impairment explore the FO more than NO. On the other hand, for a mouse with some alteration in cognitive function, the FO and NO should seem equally novel and there should be no difference between the exploration times. Another possible explanation for the fact that aged animals explore NO less may be that in mice, when receiving a new stimulus, a stress response is provoked that affects their behavior, even when they are young, but increases with aging [20].

The results obtained in the MWM (Figure 4) demonstrated the cognitive decline in the group of aged animals regarding the latency period during the 3 days of the acquisition phase, since these animals delayed significantly more time to visit the platform quadrant for the first time. The MWM indicated that old mice acquired place learning more slowly than young ones, as assessed by the evolution of escape latencies over the 3 training days. The

aged mice seemed to have a decreased ability to use spatial information; indeed, old mice need more time to see or to select and analyze the relevant cues. This indicates that both aged and young mice were able to learn where the platform was located, but old mice were slower. The fact that aged animals had the worst performance in relation with this variable has been described in the literature and R. D'Hooze and P. P. De Deyn (2001) [21] 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. Thus, the OF1_{cenp} aged mice showed impaired acquisition of the spatial learning task.

As a result of the analysis of the time in each quadrant (Figure 4A) in the second phase of the test, the presence of cognitive deterioration was confirmed in the aged mice. The time that young mice were swimming in the platform quadrant, exceeded 25% of the total time. In contrast, although aged mice spent more time in other ones. The mice of 15-16 months of age were unable to distinguish the platform quadrant clearly. The greater permanence in the platform quadrant by the animals of the young control group showed 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 [21].

Therefore, based on the different mechanisms of memory that is evaluated in the Y-maze test, MWM and in NOR, it can be affirmed that aged male OF1_{cenp} mice of 15-16 months show cognitive impairment related with age. However, in the Y-maze and novel object recognition tests, an evident cognitive decline was obtained in the 7-8-month-old mice, which implies that these memory mechanisms are affected from this age. Added to this is the behavior of the variable time spent in each quadrant in the MWM, which was similar between the 7-8 and 15-16 month-old mice.

In the case of the finding of a decrease in brain size (Figure 5A) in mice aged 7-8 and 15-16 months, it has been found *post-mortem* that the size of the brains of people with dementia is smaller than that of healthy people of the same age [22]. The decrease in brain weight might be associated with neuronal death described in dementia [23].

In the case of liver (Figure 5B), although references have been found to studies carried out in aged laboratory animals that demonstrate a relationship between their increase in size and aging [1]; these differences appear to be indistinguishable for OF1_{cenp} mice at these age.

The increase in serum glucose levels, which have been obtained in this study with OF1_{cenp} aged mice (Figure 6A), correspond to what has been reported in the literature. It has been shown that during the natural aging process that leads to cognitive decline, a hypometabolism of glucose is established in the brain, as well as glycolysis and mitochondrial function [24]. This physiological situation leads to elevated serum levels of this molecule. Although only in the 15-16 month aged animals significant differences were observed in the 7-8 month old animals there was a tendency to increase the levels of this molecule.

In relation to triglyceride and cholesterol levels according to Araki et al. (2004) [25] who compared young mice from 6-8 months and aged from 24-28 months of age, in aged animals the mobilization of lipids deteriorates with age due to the decrease in the gene expression of the transporter proteins known as Apos. This leads in the long term to an excessive accumulation of lipids in tissues such as the liver, adipose tissue and blood vessels and resulting in a higher incidence of age-related diseases. Therefore, the results obtained in this work correspond to what was stated by these authors, because the serum levels of both molecules increased significantly in OF1_{cenp} mice at the age of 15-16 months (Figure 6B, 6C). Regarding triglyceride levels, a significant increase was obtained from 7-8 months of age.

Achieved results showed that oxidative damage to lipids in brain homogenate was significantly lower in the young control group than in both groups of aged animals (Fig. 7A) and constitute another evidence about the effect of aging in mice [26]. The excess of free radicals and the special susceptibility of nervous tissue to oxidative damage, make it more vulnerable to attack by lipid membranes, proteins, RNA and nuclear DNA. Different biochemical markers related to oxidative stress such as the products of glycoxidation, lipid peroxidation, and protein oxidation, among others, have been found in higher concentration in the brain of people with cognitive decline and dementia compared to that of healthy people [27]. On the other hand, the increase in oxidative damage to proteins in the brain homogenate (Fig. 7B) corresponds to what is stated in the literature that describes it as a physiological event associated with aging [28]. Furthermore, oxidative damage to proteins could be a crucial factor in dementia due to oxidized proteins lose structural and catalytic integrity, and therefore are more susceptible to being hydrolyzed [29].

In the determination of the total antioxidant capacity (Figure 7C) the opposite was obtained, from the age of 7-8

months the tendency to decrease this capacity is shown, being significant at 15-16 months of age of the mice. This makes it possible to show that this ability is lost in animals as they age. This parameter is considered a reliable indicator of the antioxidant content of the diet and is associated with a lower risk of chronic diseases. The antioxidant buffer system can be indirectly evaluated as a total antioxidant capacity. This parameter can offer an idea of how the whole antioxidant response is to each oxidative aggressor in each system [30].

5. Conclusions

In this work, experimental evidence is compiled of the utility that naturally aged mice of OF1_{cenp} strain of 15-16 months age can have as a cognitive decline model induced by age. This evidence is based in the presence of cognitive impairment by different behavioral tests, the decrease in brain size and the increase in oxidative damage at the level of brain proteins and lipids. Also, added to this, there is the increase in serum levels of the hematological parameters analyzed.

Although not all the markers were modified from the age of 7-8 months, it can be considered that these animals could be used as a model of aging because learning and/or memory are affected in each of the three tests used. In addition, a trend was observed in the parameters that did not show significant differences to behave similarly to the animals aged 15-16 months of age. We, therefore, conclude 7-8 and 15-16-month-old OF1_{cenp} mice can be used as a model to study age-induced cognitive alterations. It is more advantageous to use aged mice with the minimum possible age because with very old animals the experiment becomes more expensive. On the other hand at very advanced ages, animals are less motivated to participate in behavioral tests and other pathologies associated with the aging process appear that could affect the usefulness of the animal model.

It is very important in the field of biomedical research, especially in preclinical studies, that researchers have several well-characterized animal models so that they can choose the one that best suits their research needs.

The correlation of the results obtained in relation to behavioral tests, hematological and biochemical analyzes indicate that OF1_{cenp} mice aged 15-16 months are a useful model of cognitive impairment as the main antecedent to dementia, and that it can be used to characterize this disorder and evaluate nutritional supplements for its prevention.

It is recommended to histologically analyze different tissues of these animals to determine their possible utility as a model of dementia.

6. List of abbreviations

ACD: Age-related cognitive decline
 CENP: CENPALAB
 NOR: Novel object recognition task
 PI: Preference index
 FO: Familiar object
 NO: Novel object
 MWM: Morris water maze
 TBARS: Thiobarbituric acid reactive substances
 SD: Standard deviation

7. 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

Funding information is not applicable/No funding was received.

-Authors' contributions

Nashelly Esquivel Crespo and Yenela García Hernández contributed equally to this work.

Nashelly Esquivel Crespo and Yenela García Hernández designed and performed experiment, organized and

analyzed data, wrote and correct the manuscript. Osmany Carvajal Hernández managed general research and drafting. Claudio Rodríguez Martínez managed general research, revised, corrected and approved the manuscript as project leader. All authors read and approved this final manuscript.

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