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Cognitive profile of male mice exposed to a Ketogenic Diet

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ABSTRACT

In recent years, nutritional interventions for different psychiatric diseases have gained increasing attention, such as the ketogenic diet (KD). This has led to positive effects in neurological disorders such as Parkinson's disease, addiction, autism or epilepsy. The neurobiological mechanisms through which these effects are induced and the effects in cognition still warrant investigation, and considering that other high-fat diets (HFD) can lead to cognitive disturbances that may affect the results achieved, the main aim of the present work was to evaluate the effects of a KD to determine whether it can induce such cognitive effects. A total of 30 OF1 male mice were employed to establish the behavioral profile of mice fed a KD by testing anxiety behavior (Elevated Plus Maze), locomotor activity (Open Field), learning (Hebb Williams Maze), and memory (Passive Avoidance Test). The results revealed that the KD did not affect locomotor activity, memory or hippocampal-dependent learning, as similar results were obtained with mice on a standard diet, albeit with increased anxiety behavior. We conclude that a KD is a promising nutritional approach to apply in research studies, given that it does not cause cognitive alterations.

1. Introduction

In recent years, it has been widely discussed whether dietary modifications may be an important factor in several diseases [1,2]. Many of these new interventions modify one or more macronutrients, such as high-fat diets, low-carbohydrate or low-sugar diets [3,4]. An example that has been recently explored as a therapeutic target is the ketogenic diet (KD), which leads to changes in the body's own metabolism [5,6]. The KD has traditionally been used in epilepsy [7,8], but lately, it has also been used as a nutritional intervention to investigate its effects in other neurological disorders, such as Alzheimer's disease [9], Parkinson's disease [10], autism [11] and, most recently, addiction [12,13]. However, more research with randomized control trials is required to provide conclusive results.

The KD is a diet high in fat, low in carbohydrates and moderated in proteins. One of the main features of the KD is the reduction in carbohydrate intake, which reduces the production of glucose and induces the body to use fat stores, breaking down fatty acids and creating ketone bodies in the liver, like β-hydroxybutyrate (βOHB) [7,14]. This metabolic process is known as ketosis, which can be achieved by strict adherence to a KD [15], or by prolonged fasting [16,17]. βOHB is a non-volatile and stable compound released into the bloodstream [18] and constitutes up to 70% of the ketone bodies synthesized in liver mitochondria [19], being the main indicator of ketosis both in humans [13] and in animal models such as rats [20] and mice [12,21].

To date, numerous studies with high-fat diets have reported negative effects on behavior, such as locomotor activity [22,23], or cognition, such as learning deficits [24–27], impaired hippocampus-dependent memory [23,25,28,29] and even anxiety-like behavior [30]. These effects are crucial in animal model research, as a subtle deficit in these capabilities could be interfering with the results obtained on many levels. Sometimes, certain behavioral procedures in preclinical research require the animal to learn a task or remember an object. If dietary treatments like HFDs affect behavior and cognition, we could be attributing benefits, harms, or no significant results to other causes and not to the diet itself, leading us to contaminated conclusions.

Therefore, as the KD is increasing its popularity in research in several neurological diseases, such as anxiety, depression, bipolar disorder or attention deficit hyperactivity disorder [31], it is still necessary to establish a baseline of the behavioral and cognitive profile of the chronic

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administration of this type of diet in order to confirm if it is safe or, on the contrary, if it has similar effects to those of traditional high-fat diets [21].

As mentioned above, the main difference between the KD and HFDs is that traditional high-fat diets not only contain an important percentage of carbohydrates, with a significant proportion of sugar, but also contain fats obtained from lard and soybean oil. In previous behavioral studies, it has been reported that some of the main factors contributing to cognitive alterations are the metabolic effects of HFD exposure, such as increased fat intake and body weight gain and the general metabolic dysfunction, with alterations in insulin, ghrelin and leptin levels [24, 32–34].

Therefore, the aim of the present work was to evaluate the cognitive and behavioral differences observed in young and cognitively healthy mice exposed to a KD to explore the effects of this diet in standard animals. For this purpose, we provided the KD to OF1 mice and evaluated the differences in their anxiety behavior with the Elevated Plus Maze, their spontaneous locomotor activity using the Open Field test, their memory with the Passive Avoidance test and their hippocampal learning with the Hebb-Williams maze.

2. Material and methods

2.1. Subjects

A total of 30 male mice of the OF1 outbred strain were acquired commercially from Charles River (France). Animals were 21 days old on arrival at the laboratory and were all housed under standard conditions in groups of 4 (cage size 28 × 28 × 1 4.5 cm) for three days prior to initiating the experimental feeding condition (PND 25), at a constant temperature (21±2 °C), lights on from 8:00 to 20:00, and food and water available ad libitum (except during the behavioral tests). All procedures involving mice and their care complied with national, regional and local laws and regulations, which are in accordance with Directive 2010/63/EU of the European Parliament and the council of September 22, 2010 on the protection of animals used for scientific purposes. The Committee for the Use and Care of Animals of the University of Valencia approved the study (2019/VSC/PEA/0065). The size of the sample was determined with the G*Power program [35], estimating the need to include 13 mice per experimental group. An expected effect size of $d = 1.5$ ($\alpha = 0.05$ and statistical power = 0.95) was taken, based on the results of the previous study of Blanco-Gandía et al. [24], which employed a similar experimental design, strain of mice, age of the animals and behavioral tests.

2.2. Apparatus and procedure

2.2.1. Feeding conditions and experimental design

Two different types of diet were used in this study: the standard diet (SD) (Teklad Global Diet 2014, 13 kcal% fat, 67 kcal% carbohydrates and 20% kcal protein; 2,9 kcal/g) and the ketogenic diet (KD) (TD.96355, 90.5% kcal from fat [vegetable shortening and corn oil], 0.3% kcal from carbohydrates and 9.1% kcal from protein; 6.7 kcal/g). The different diets were supplied by Envigo Teklad Diets (Barcelona, Spain). In this experiment (Fig. 1a), OF1 male mice ($n = 30$) arrived in the laboratory on PND 21 and were randomly divided into 2 groups ($n = 15$/condition) with similar average body weights (25–26 g) and assigned either SD or KD feeding conditions. Tests were performed one week after the diet had been initiated in order to evaluate if it had induced alterations in anxiety behavior (Elevated Plus Maze on PND32), motor activity (Open Field on PND 33), memory (Passive Avoidance Test on PND 34) or learning (Hebb Williams Maze on PND 36). All animals were under their specific feeding conditions from one week before the behavioral tests began (PND 25) until the end of the experiment (PND 44). On PND 44, the KD group was switched back to the SD until the end of the experiment to reevaluate anxiety behavior with the standard diet (PND 51). Body weight and Beta-hydroxybutyrate (βOHB) plasma levels were measured every week throughout the study.

2.2.2. Ketosis status: β-hydroxybutyrate plasma levels

Plasma β-hydroxybutyrate was measured weekly from the tail vein with a On Call GK Dual monitor and ketone test strips (ACON Laboratories, Inc., San Diego, CA).

2.2.3. Elevated plus maze

The EPM consisted of two open arms (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 15 cm). The junction of the four arms formed a central platform (5 × 5 cm). The floor of the maze was made of black Plexiglas, and the walls of the enclosed arms of clear Plexiglas. The open arms had a small edge (0.25 cm) to provide additional grip for the animals. The entire apparatus was elevated 45 cm above floor level. In order to facilitate adaptation, mice were transported to the dimly

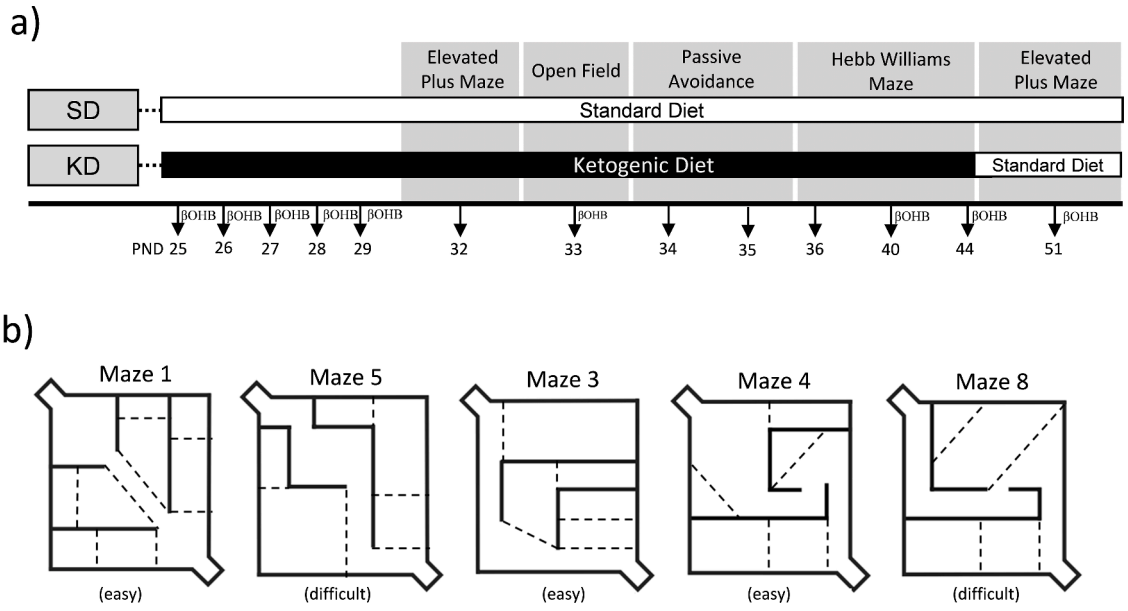

Fig. 1. (a) Experimental design. (b) Hebb-Williams Maze configuration and difficulty.

illuminated laboratory 1 h prior to testing. At the beginning of each trial, subjects were placed on the central platform facing an open arm, and were allowed to explore for 5 min. The maze was thoroughly cleaned with a damp cloth after each trial. The behavior displayed by the mice was recorded automatically by an automated tracking control software (EthoVision 3.1; Noldus Information Technology, Leesburg, VA). The measurements recorded during the test period were frequency of entries, time and percentage of time spent in each section of the apparatus (open arms, closed arms, central platform). An arm was considered to have been visited when the animal placed all four paws on it. Number of open-arm entries, time spent in open arms and percentage of open-arm entries are generally used to characterize the anxiolytic effects of drugs [36,37].

2.2.4. Open field

The spontaneous locomotor behavior of the mice was quantified in an Open Field for a period of 1 hour. The Open Field test was performed in an opaque plastic box (30 × 30 × 15 cm) left open at the top. The animal was placed in the box and its activity was recorded automatically by tracking software (EthoVision 3.1; Noldus Information Technology, Leesburg, VA). The parameter studied was the total distance traveled (cm) and time near the wall and center (s).

2.2.5. Passive avoidance test

For the Passive Avoidance test, a step-through inhibitory avoidance apparatus for mice (Ugo Basile, Comerio-Varese, Italy) was employed. This cage is made of Perspex sheets and divided into two compartments (15 cm × 9.5 cm × 16.5 cm each one). The safe compartment is white and illuminated by a light fixture (10 W) fastened to the cage lid, whereas the "shock" compartment is dark and made of black Perspex panels. The two compartments are divided by an automatically operated sliding door at floor level. The floor is made of 48 stainless steel bars with a diameter of 0.7 mm and situated 8 mm apart.

Passive Avoidance tests were carried out following the procedure described in Aguilar et al. [38]. On the day of training, each mouse was placed in the illuminated compartment facing away from the dark compartment. After a 60 s period of habituation, the door leading to the dark compartment was opened. When the animal had placed all four paws in the dark compartment, a footshock (0.5 mA, 3 s) was delivered and the animal was immediately removed from the apparatus and returned to its home cage. The time taken to enter the dark compartment (step-through latency) was recorded. Retention was tested 24 h later, following the same procedure but without the shock. The maximum step-through latency was 300 s.

2.2.6. Hebb-Williams maze

The maze used in our experiment is made of black plastic and measures 60 cm wide x 60 cm long x 10 cm high. It contains a start box and a goal box (both 14 cm wide x 9 cm long), which are positioned at diagonally opposite corners. The maze contains cold water at a wading depth (15 °C, 3,5 cm high), while the goal box is stocked with fresh dry tissue. Several maze designs are produced by fixing different arrangements of barriers to a clear plastic ceiling. This apparatus allows the cognitive process of routed learning and the motivation of water escape to be measured.

The procedure followed was based on that employed by Galsworthy et al. [39], in which mice must navigate the maze and cross from the wet starting box to the dry goal box in order to escape the cold water. Animals underwent a 5-min habituation period (dry sand, no barriers) on day 1, and undertook problem A on day 2 and problem D on day 3 (4 trials/day) (practice mazes). Mice were subsequently placed in mazes 1, 5, 3, 4 and 8 on separate days (Fig. 1b), on which 8 trials took place (see Rabinovitch & Rosvold [40] for all maze designs). The time limit for reaching the goal box was 5 min, after which the mouse was guided to the box if necessary. The total latency score (sum of the latencies in all the problem trials in each maze) was registered.

2.3. Statistical analysis

Data relating to βOHB and body weight were analyzed by a mixed ANOVA with one between-subjects variable – "Diet", with 2 levels, (SD and KD) - and a within variable – "Days", with 9 (Baseline, Days 1–4, 7, 15, 19 and 25) or 4 levels (Baseline and weeks 1–3).

Data relating to the Elevated Plus Maze and Open Field test were analyzed by a one-way ANOVA with a between variable - "Diet", with 2 levels for Open Field (SD and KD) and three levels for the Elevated Plus Maze test (SD, KD and 7 days post-KD). The Passive Avoidance test was analyzed by a two-way ANOVA, with the same between variable and one within variable - "Days", with 2 levels (training day and test 24 h). The data of the Hebb-Williams maze were analyzed by a two-way ANOVA with one between subject variable – "Diet" - and one within subject variable – "Maze", with five levels. The Bonferroni adjustment was employed for post hoc comparisons. All results are expressed as mean ± S.E.M. Analyses were performed using SPSS v26.

3. Results

3.1. β-hydroxybutyrate (βOHB) and body weight

The ANOVA of the βOHB plasma levels (Fig. 2a) revealed a significant effect of the interaction "Days x Diet" [$F(8224) = 25{,}594$; $p < 0.001$], as the KD group showed increased levels of βOHB in comparison with the SD group at 24 h, 48 h, 72 h, 96 h, 7, 15 and 19 days ($p < 0.001$ in all cases), but showed no differences at 25 days ($p = 0.195$). There were also significant increases within the KD group on Days 1, 2, 3, 4, 7, 15 and 19 with respect to baseline ($p < 0.001$ in all cases), but no differences with respect to 25 days.

The ANOVA of body weight (Fig. 2b) revealed a significant effect of the interaction "Week x Diet" [$F(3,84) = 14{,}364$; $p < 0.001$] as mice on the KD displayed lower body weight at baseline and during week 1 compared to weeks 2 and 3 ($p < 0.001$, in all cases). Mice fed a SD showed higher body weight in Week 3 compared to Baseline, and Weeks 1 and 2 ($p < 0.001$, in all cases). In addition, the SD group exhibited higher body weight than the KD group at Baseline ($p < 0.05$) and in Week 1 ($p < 0.01$).

3.2. Elevated plus maze

The ANOVA (see Table 1) revealed an effect of the variable "Diet" for the time [$F(2,42) = 4.838$; $p = 0.013$] and percentage of time [$F(2,42) = 4.944$; $p = 0.012$] spent in the open arms of the maze. Animals belonging to the KD group spent less time and percentage of time in the open arms than the SD group ($p < 0.01$ in both cases).

There was also a significant effect of the variable "Diet" for the percentage of open entries [$F(2,42) = 3.317$ $p = 0.046$]. The KD group showed a lower percentage of open entries than the SD group ($p < 0.05$). There was no effect of the variable Diet on the total number of entries [$F(2,42) = 0.428$; $p = 0.655$]. There were no significant differences between the SD group and 7 days post-KD.

3.3. Open field

The ANOVA of the Open Field (Fig. 3 and Table 2) revealed no significant differences in the variable "Diet" for the distance traveled [$F(1,18) = 0.175$; $p = 0.681$], time near the wall [$F(1,18) = 0.731$; $p = 0.404$] and time in the center [$F(1,18) = 0.038$; $p = 0.848$].

3.4. Passive avoidance test

The results of the Passive Avoidance test are presented in Fig. 4. The ANOVA revealed an effect of the variable "Days" [$F(1,28) = 146.006$; $p < 0.001$], with all the groups presenting longer step-through latencies in the 24 h test with respect to the training session ($p < 0.001$). The ANOVA

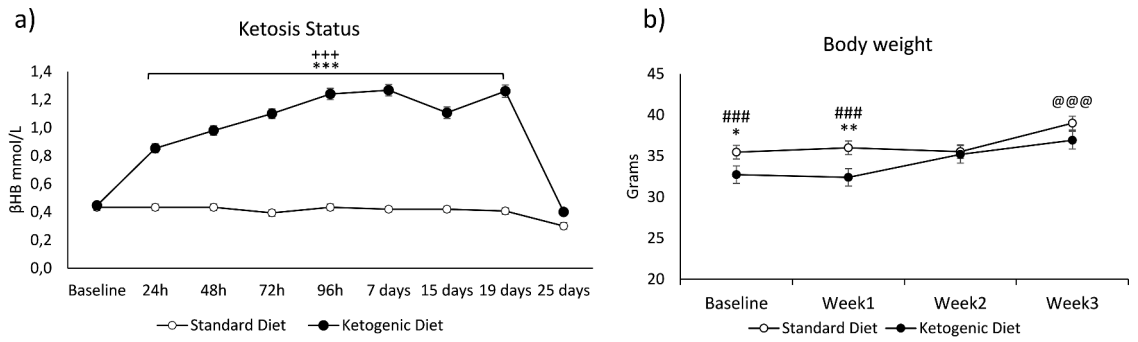

Fig. 2. β-hydroxybutyrate plasma levels and weekly body weight. (a) Ketosis status. Data are represented as the mean (±SEM) amount of βOHB. **(b) Weekly body weight**. Data are represented as the mean (±SEM) body weight measured weekly. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ significant difference with respect to the SD group. +++$p < 0.001$ significant difference with respect to baseline. ### $p < 0.001$ significant difference with respect to week 2 and 3 of KD. @@@$p < 0.001$ significant difference with respect to the rest of the weeks of SD.

Table 1
Effects of a KD on male mice in the Elevated Plus Maze ($n = 15$/group). Data are presented as mean values ± S.E.M. *$p < 0.05$; **$p < 0.01$ significant difference with respect to SD.

	Standard Diet	Ketogenic Diet	7 days post-Ketosis
Time OA	67 ± 15	20 ± 3 **	50 ± 11
% Time OA	33 ± 6	12 ± 2**	24 ± 5
% Open Entries	50 ± 4	31 ± 6*	43 ± 5
Total Entries	37 ± 4	37 ± 3	33 ± 4

did not show significant differences for the variable "Diet" [F(1,28)= 0.335; $p = 0.567$] or the interaction "Days x Diet" [F(1,28)=0.017; $p = 0.896$]. All animals remembered the footshock of the training session.

3.5. Hebb-Williams maze

The ANOVA for the total latency score (Fig. 5) revealed an effect of the variable "Maze" [F(4,96)=10.456 ; $p < 0.001$]. Maze 1 was significantly easier for all the groups than mazes 3, 4 and 5 ($p < 0.001$ in all cases) and 8 ($p < 0.01$), as the animals took less time to reach the goal. There were no significant differences in the variable "Diet" [F(1,24)= 0.040; $p = 0.843$] or the interaction "Maze x Diet" [F(4,96)=0.406; $p = 0.804$].

Table 2
Effects of a KD in the time (s) near the wall, center, rest of the field and total time in the Open Field by mice ($n = 10$/group). Data are presented as mean values ± S.E.M.

	Near the Wall	Center	Rest of the field	Total time
Standard Diet	1283 ± 42	519 ± 41	1798 ± 11	3600
Ketogenic Diet	1334 ± 47	510 ± 29	1756 ± 28	3600

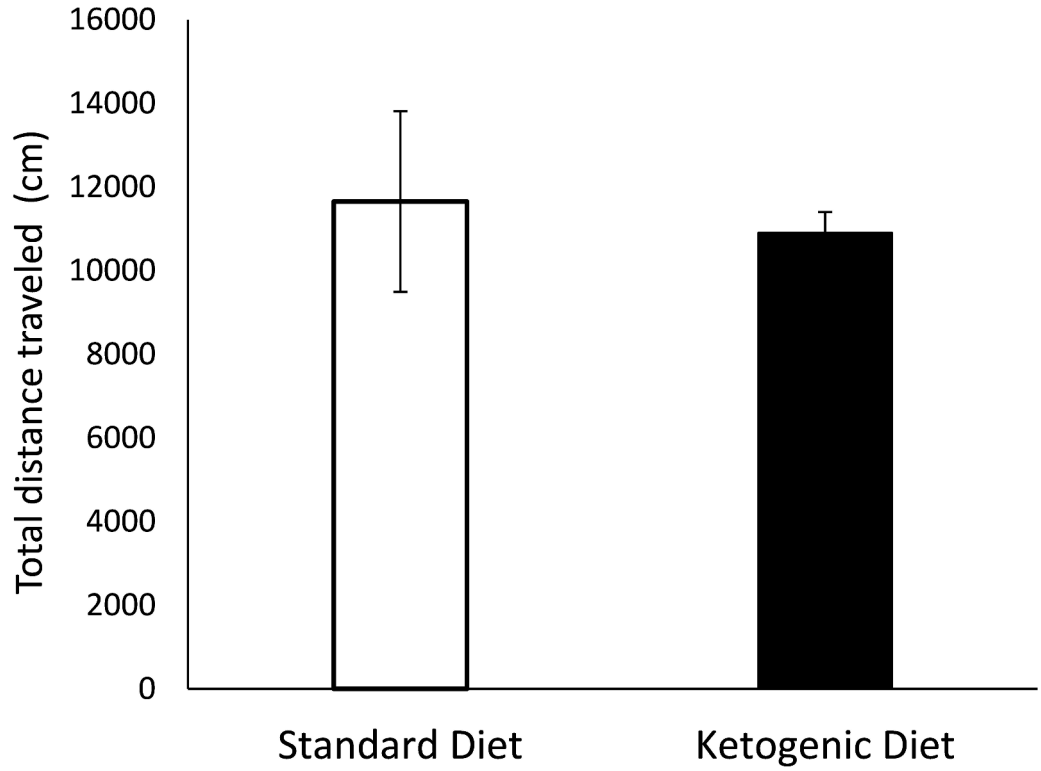

Fig. 3. Effects of a KD on the total distance covered in 1 h in the Open Field by mice ($n = 10$/group). Data are presented as mean values ± S.E.M.

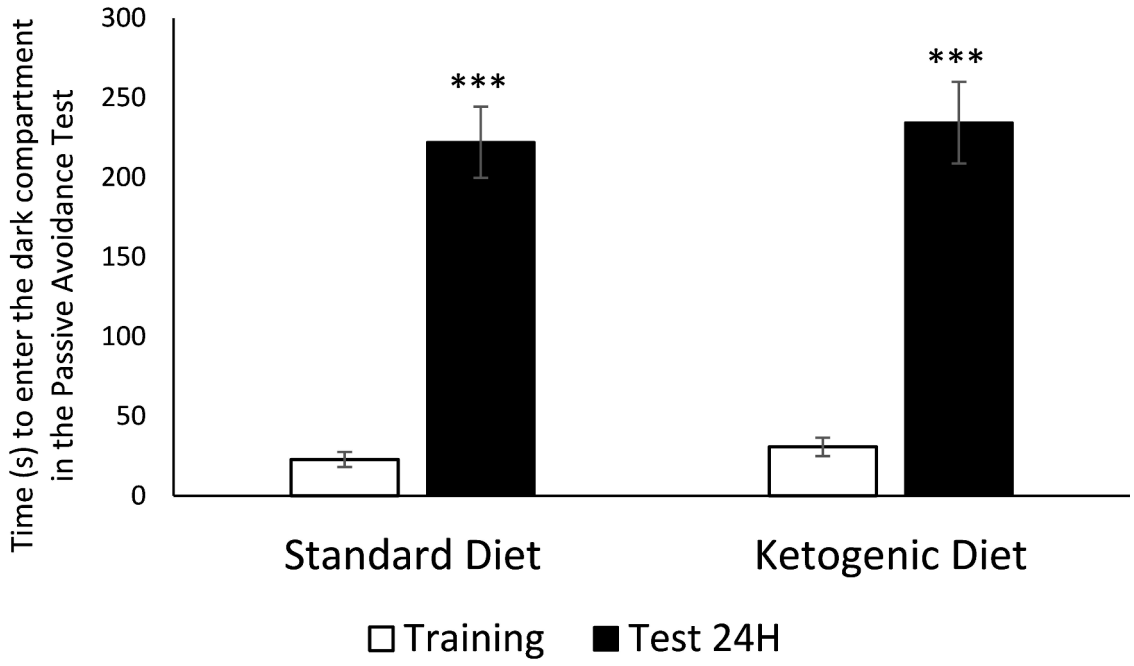

Fig. 4. Effects of a KD on the time taken for male mice to enter the dark compartment in the Passive Avoidance test during training and 24 h after training ($n = 15$/group). Data are presented as mean values ± S.E.M. *** $p < 0.001$ significant differences with respect to training.

Fig. 5. Effects of a KD on the total latency score to reach the goal in the 8 trials of male mice in the Hebb-Williams maze. The mazes were classified as easy (1, 3 and 4) or difficult (5 and 8). ($n = 15$/group) ***$p < 0.001$ significant difference with respect to Maze 3, 4 and 5; ++ $p < 0.01$ significant difference with respect to Maze 8. Data are presented as mean values ± S.E.M.

4. Discussion

The ketogenic diet has been assessed to determine if it can be used as a nutritional treatment for numerous pathologies, such as epilepsy, Parkinson's disease, Alzheimer's disease, cancer, and more recently, drug addiction [7,9,13]. Although it has been observed that the KD could be an effective treatment option in certain pathologies, such as in children with drug-resistant epilepsy [41], further research is needed to achieve conclusive results in neurological disorders. Preclinical research in these fields studies the possible effects of nutritional interventions, and to draw conclusions, it is necessary to combine physiological results with behavioral outcomes. Experimental treatments, context or researchers can interfere with animal behavior, sometimes leading to inaccurate research conclusions.

As stated in the introduction, the main aim of the present work was to address whether the KD per se had any effects on anxiety, locomotor activity, memory, and learning. Our results confirmed that, even when the KD altered the animal's metabolism, with the increase in ketones,

this diet did not affect the cognitive profile of mice, as no significant changes were observed in locomotor activity, memory, or hippocampal-dependent learning with respect the group fed a standard diet. Interestingly, animals on a KD showed an increase in anxiety behavior 7 days after beginning the KD regimen.

Results related to metabolism confirmed that animals exposed to a KD rapidly displayed a ketotic state, as ketone body levels of βOHB significantly increased from the first 24 h onwards. When the individual is in a ketotic state, the reduction of carbohydrates in the diet leads to a drastic reduction in the levels of glucose, which ceases to be available as the main source of energy, inducing the body to generate ketone bodies in the liver [42,43]. This rise in blood ketone bodies induces a different metabolism [15] in which ketones, rather than sugar, become the main source of energy. In our study, these values remained stable while the animals were fed with the KD, but returned to normal 24 h after they were switched back to the SD. βOHB levels are widely used as a biomarker of ketosis in mice and humans [15], and our results are consistent with other studies that indicate that KD increases βOHB levels in both mice [21] and rats [20]. With respect to body weight and the KD, preclinical studies have revealed some disagreement, as different studies suggest that a KD can increase or decrease body weight in rats [12, 44–46]. This may be due to methodological issues, as it is necessary to match eucaloric diets. In our study, even when animals rapidly entered a ketotic state, their body weight did not differ from that of animals exposed to the SD, which also corresponds with previous results [21,47].

Our results showed that animals fed with the KD spent less time and percentage of time in the open arms of the EPM and made a lower percentage of open entries than those fed with the SD. This result is in contrast with the only other study that evaluated anxiety in animals fed on a KD, which reported no changes with respect to animals fed with a SD [21]. Another study reported that 8 weeks of ketone supplementation also did not induce any changes in anxiety [48]. A plausible explanation for the result obtained in our study is that, in every previous study, anxiety was evaluated 3 months after being on the KD and 8 weeks after supplementation, while we measured anxiety only 7 days after the beginning of KD administration. Adenosine receptors in GABAergic neurons play an essential role in anxiety regulation [49,50] and KD induces modifications in the adenosinergic systems [51], which could explain these initial alterations. The increase in anxiety observed in our study could be due to the short time of habituation to this type of diet. We confirmed that this increase in anxiety was due to the KD, as anxiety levels returned to normal when animals were switched back to the SD. To confirm the results obtained in the EPM, we assessed the time spent near the wall in the Open Field test and found no significant differences between both groups. This may be due to the fact that the EPM is much more sensitive [52], and that the EPM and the Open Field tests can measure different aspects of anxiety [53]. Indeed, several studies have reported symptoms of anxiety in mice using the EPM, but not with the Open field test [54]. Combining our results with those of previous studies, we could hypothesize that anxiety symptoms produced by the KD would be reduced over time. This result is an important issue to consider in future studies, as it indicates that it is prudent to lengthen the diet adaptation phase before the beginning of any behavioral test. For example, anxiety levels may be interfering if the animal is unable to complete the task of exiting a maze, and the researcher may misattribute this behavior to a lack of learning ability.

Focusing on the cognitive profile and the possible effects of a KD, no changes were observed in locomotion, learning or memory, with the exception of anxiety. In this line, the results of the present work showed that mice on the KD displayed similar locomotion abilities to animals fed the SD when evaluated in the Open Field test, which suggests that the KD does not alter locomotion behavior. The Open Field is a commonly used test, and several studies have confirmed that a KD does not affect general locomotor activity in rats [55,56] and mice [57]. Similar results have been reported by studies evaluating locomotor activity in mice receiving a ketone supplementation [48]. This result could be novel and promising, as studies employing common HFDs have reported alterations in locomotion, such as hyperlocomotion [24,58], or a decrease in activity [59,60]. This outcome would be crucial in studies on pathologies like epilepsy or Parkinson's disease, where a locomotor alteration may mask the real effects of nutritional interventions [10,61].

Regarding implicit memory, independently of the dietary treatment, all animals presented longer step-through latencies in the 24 h test with respect to the training session, confirming that they remembered the footshock received earlier. These results confirm those of a previous study in which a KD did not affect a contextual fear-conditioning task in rats [62].

In the same line are the results obtained in hippocampal-dependent learning, which showed that the KD group required the same time as the SD group to reach the goal in all the mazes, confirming that a KD does not alter acquisition of learning. Mice fed with the SD and the KD spent a similar amount of time in the easy or difficult (5) mazes. In general, difficult mazes can discriminate between groups when there is a cognitive deficit in any of them. In the present study, after 8 trials, regardless of diet, all mice always learned the task. A possible explanation for the reduction of time needed to complete Maze 8 despite being considered a difficult maze is the experience that had been previously acquired. The day before performing Maze 8, all mice completed Maze 4, which presents a comparable configuration (Fig 1b) and thus were familiarized with the spatial configuration and learned the task comparatively faster. The Hebb-Williams maze is a very sensitive test which is employed to detect spatial learning deficiencies, but there are no studies to date employing this maze to test KD effects. However, results from other studies have shown no deficits in the Y-maze or Water Maze in male mice kept on a KD for 3 months [21], or in the Novel Object Recognition test [63]. This result is in contrast with previous studies on common HFDs, where it has been shown that continuous exposure to a HFD induces marked memory and spatial learning deficits [24,29,64–67]. This affection might be triggered by leptin levels, which are significantly increased with HFDs [24,68]. It has been shown that when leptin levels increase, memory and learning can be affected [29, 69]. In fact, recent studies have suggested that not only does the KD not produce learning and memory impairments but it rescues hippocampal memory deficiencies in mice presenting impairments caused by age [70] or rats exposed to chronic stress environments [71]. Although the KD has different characteristics and metabolic properties from the common HFDs employed to date, it is still a high-fat diet. Thus, it is necessary to confirm the lack of detrimental effects induced by it. Conventional animal HFDs contain approximately 30–40% of carbohydrates, of which approximately 20% are sugars; while in the KD, the main component is fat (90%), followed by protein and less than 5% carbohydrates. These differences in sugar and carbohydrate composition are the main explanation for the physiological effects of KDs on several diseases. Although both diets are high in fat, the changes that they produce in metabolic status are significantly different.

In preclinical studies, anxiety and locomotor behavior are both commonly used as behavioral complements in several areas of knowledge. In addition, learning and memory are most employed in areas of neurodegenerative disorders and drug addiction, areas in which the KD is increasingly becoming a focus of interest [9,13]. A pharmacological or nutritional treatment can have a significant physiological effect (cells, metabolism), but this effect might not be reflected in a behavioral improvement. For example, a specific treatment could clear amyloid beta protein without an improvement in the learning or memory performance of the animals. Another example could be found in models of addiction, where animals have to learn an operant task to obtain the drug (models of self-administration) [72] or make a contextual association with the drug (models of conditioned place preference), which requires memory and learning abilities for the acquisition process [73]. If the diet per se is affecting learning and memory, the researcher might draw wrong conclusions about the drug, as food, rather than the drug itself, could be influencing the animals' cognitive ability. Therefore,

preclinical models of behavior help us to confirm whether the different pharmacological or nutritional treatments are effective. To achieve this end, we must ensure that the treatment per se does not affect behavior or cognition.

5. Conclusion

The present study shows that the KD is a nutritional intervention that does not affect behavior or cognitive performance in male mice. However, one important limitation of this study has been not exploring the effects of this diet in female mice. It should be noted that understanding sex differences in the clinical application of a KD is a very relevant factor to take into account, especially given that the cognitive and behavioral consequences of some psychiatric disorders differ between males and females, as in anxiety and depressive disorders [74]. Therefore, further research is needed to know the effects of a KD in females. In addition, in this study we have evaluated the short-term cognitive effects of a KD, but a longer exposure to this diet before the behavioral testing would verify the lack of cognitive alterations.

Future studies in the addiction and neurodegenerative disease fields that are exploring nutritional interventions with the KD can safely employ different tests of memory, learning and locomotor activity, as well as anxiety, always providing a prudent habituation time to the diet, as anxiety could otherwise alter the results of other tests.

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Author contributions

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Data Availability

Data will be made available on request.

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