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Beneficial effect of ovocystatin on the cognitive decline in APP/PS1 transgenic mice

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ABSTRACT

Purpose: Cystatin C plays an important role in the course of neurodegenerative diseases and has a beneficial effect through inhibiting cysteine proteases and amyloid-β aggregation. It also induces proliferation and autophagy. Cystatin isolated from chicken egg white, called ovocystatin, has been widely used in the medical and pharmaceutical research due to its structural and biological similarities to human cystatin C. The aim of this study was to assess the effect of administering ovocystatin on the development of dementia-specific cognitive deficits in APP/PS1 transgenic mice.

Materials/methods: The study was conducted on transgenic B6C3-Tg(APPswe,PSEN1dE9)85Dbo/Mmjax mice. Ovocystatin was administered to four-month-old transgenic (AD) and wild type (NCAR) mice in drinking water for 24 weeks (at a dose of 40 and 4 μg/ mouse). The locomotor activity and cognitive functions were determined using an actimeter and the Morris water maze test, respectively.

Results: The results of the study indicate that ovocystatin has a beneficial effect on the cognitive functions in APP/PS1 transgenic mice. The strongest effects of ovocystatin were found in the group of AD mice, where ovocystatin was administered in drinking water at a dose of 40 μg/mouse ($p < 0.05$). Mice from the AD group swam statistically significantly further in the target zone during the trial in the Morris water maze compared to the AD (vehiculum) group ($p < 0.05$).

Conclusions: The obtained results encourage further research into the protective effect, which may be used as an adjuvant in the treatment of deteriorating cognitive functions.

1. Introduction

Alzheimer's disease is a major cause of dementia. It causes progressive cognitive impairment affecting learning, memory and daily functioning and may reduce the quality of life, and lead to premature death [1]. Age is one of the main risk factors for Alzheimer's disease patients [2–4]. Mutations in the genes encoding amyloid precursor protein (*APP*) and presenilin 1 and 2 (Presenilin 1 gene, *PSEN1*; Presenilin 2 gene, *PSEN2*) cause Alzheimer's Disease, leading to an increase in the production of toxic amyloid-β (Aβ). These mutations are responsible for the development of the disease in 50% of patients with early-onset autosomal dominant Familial Alzheimer's disease (FAD) [4,5]. In addition, mutations in the *PSEN1* and *PSEN2* genes increase the production of an amyloidogenic form of Aβ such as $A\beta_{42}$ [4,6]. Currently, Alzheimer's disease is treated symptomatically with cholinesterase inhibitors (donepezil, rivastigmine) and the antagonist of the N-methyl-D-aspartate (NMDA) receptor (memantine) [7,8]. There is ongoing research into new preventive and therapeutic measures in the course of Alzheimer's disease. Current studies into new therapeutic strategies have focused on the so-called "amyloid cascade" hypothesis [9]. In addition, novel immunomodulatory, neuroprotective (anti-inflammatory, anti-oxidative), anti-fibrillization and anti-aggregation (chelation of metal ions, amyloid binding ligands) therapies are sought [10].

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Over the past twenty years, the findings of genetic, experimental and clinical studies have suggested that cystatin C may play a role in Alzheimer's disease. Cystatin C is an inhibitor of cysteine proteases, it is present in all tissues and body fluids and it is encoded by the *CST3* gene. Studies have found that it exhibits a wide range of biological functions. It modulates the immune response, has an antiviral and antibacterial function and inhibits tumour metastases [11–14]. Cystatin C co-deposits with Aβ in the brains of patients with Alzheimer's disease [15]. Studies have found that cystatin C binds to APP, $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$. It also inhibits fibril formation and oligomerization depending on its concentration [16,17]. In *vivo* and in vitro studies have confirmed that cystatin C binds to soluble Aβ and prevents its deposition in APP transgenic mice expressing the *CST3* gene. The expression of twice as much human cystatin C as endogenous mouse cystatin C reduces Aβ accumulation [18]. in vitro studies indicate that cystatin C protects hippocampal neurons derived from rat brains against toxic oligomers and fibrillary forms of Aβ [19]. Apart from preventing oligomerization and the formation of β-amyloid fibrils, cystatin C inhibits cysteine proteins, induces autophagy and stimulates neurogenesis [20].

Ovocystatin, which is the first isolated cystatin, is a type 2 cystatin and is the most-studied protein from the cystatin family [21]. Ovocystatin is an inhibitor of the C1 family cysteine peptidases, which includes cathepsins B, H, K, L and S [12,22]. Ovocystatin and human cystatin C share significant structural similarity. The amino acid sequence in ovocystatin is 44% homologous to human cystatin C, and the proteins share 62–63% structural similarity. The secondary crystal and solution structure are also similar between the two proteins [23,24]. Due to the fact that the two proteins are orthologous and share similar biological properties, ovocystatin is currently used in many experimental studies [25]. It has also been reported that ovocystatin is characterized by relatively low immunogenicity [26]. Moreover, it has been recently suggested that ovocystatin has protective properties in young rats [27], as well as may prevent aging-related cognitive impairment [28]. Thus, the present study aims to evaluate the effects of treatment with ovocystatin on learning impairment and memory in APP/PS1 transgenic mice.

2. Materials and methods

2.1. Reagents and administration

Ovocystatin was isolated from egg white by means of affinity chromatography on S-carboxymethylated papain-Sepharose 4B, using a modification [29] of the method described by Anastasi et al. [21]. The expression yield and the specific activity of ovocystatin were calculated based on the antipapain activity and the concentration of the protein in an egg white homogenate and in the final product. The inhibitory activity against papain was measured using Nα-Benzoyl-DL-arginine β-naphthylamide (BANA) as a substrate [30,31]. The protein concentration was determined using a 0.871 extinction coefficient at 280 nm for a 0.1% ovocystatin solution [21]. The purity of ovocystatin was determined by gel filtration on an HPLC system using a Bio Sec-5 column (Agilent Technologies), and the purity always exceeded 95% (Fig. 1). Specific antipapain activity of the purified inhibitor ranged from 20 to 25 U/mg protein. The final product was lyophilized as described by Gołąb et al. [32].

Ovocystatin was administered in drinking water (in glass drinking tubes) containing 0.1% albumin from bovine serum (Fluka) that acted as a stabiliser [33,34]. The mice received ovocystatin solutions according to the groups described below. The vehiculum groups received only drinking water containing 0.1% of bovine serum albumin (*vehiculum*). Starting at 17 weeks of age, the animals received the solution every 24 h. Behavioural tests were carried out after 22 weeks of ovocystatin administration.

2.2. Animals and experimental design

The study was carried out using 17-week-old male *B6C3-Tg (APPswe,PSEN1dE9)85Dbo/J* transgenic mice (AD, initial body weight approximately 25.0 g, n = 27) and wild-type mice (NCAR, initial body weight approximately 24.5 g, n = 25) (Jackson Laboratory, Bar Harbor, USA). Transgenic mice express the mouse/human APP_{swe} (K595 N/M596 L) and exon-9 deleted presenilin 1 (deltaE9). In this murine model, the first Aβ depositions develop by six months of age [35,36]. The mice were kept (3–4 per cage) in conditions with a 12:12 h light-dark cycle (lights on at 7:00 a.m.) at a temperature of 21 ± 2 °C and free access to food and water.

The animals were divided into six groups: 1. AD (vehiculum; n = 9) 2. AD + CYS40 (40 μg/mouse; n = 9), 3. AD + CYS4 (4 μg/mouse; n = 9), 4. NCAR (vehiculum; n = 8). 5. NCAR + CYS40 (40 μg/mouse; n = 9), 6. NCAR + CYS4 (4 μg/mouse; n = 8). The animals were weighed at the beginning of the study and in the first, second, third, fourth and fifth month of administering the substances as well as before and after carrying out the behavioural tests.

2.3. Locomotor activity recording

Locomotor activity was assessed using an IR Double IR System for Mice (PanLab, Spain) actimeter for locomotor activity. The mice were placed in the actimeter chamber one by one and were observed for 5 min. The actimeter chamber was cleaned after each animal with an alcohol-based agent. SEDACOM software was used to determine slow movements (PanLab, Spain). Locomotor activity was tested at three time points: prior to the administration of ovocystatin, 12 weeks after initiating the therapy and prior to initiating the Morris water maze (MWM) test in order to rule out any bias caused by age related diseases.

2.4. Morris water maze – spatial learning test

A round white pool 122 cm in diameter with a 25 cm high side wall was filled with tap water (maintained at 22 °C) up to approximately 21 cm (day 1) and up to 19 cm on the following days (days 2–5) and a 20 cm platform was used. The water was coloured white with UHT milk for contrast. The water in the pool was drained and replaced daily.

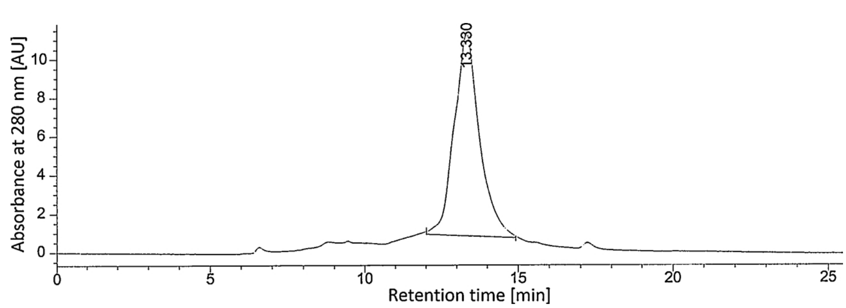

Fig. 1. Characterization of the final ovocystatin preparation by gel filtration on an HPLC system using a Bio SEC-5 column. The purity of the preparation was greater than 95%.

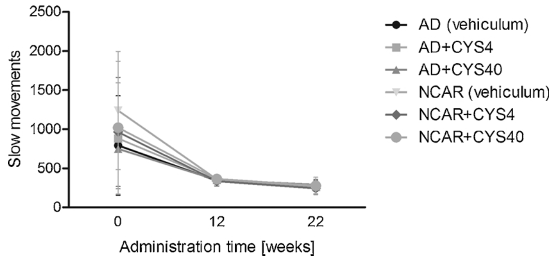

Fig. 2. The influence of administering ovocystatin on locomotor activity. Data are expressed as mean ± SD of slow movements.

Visual cues in the form of flags were erected on each side of the pool and on the two curtains surrounding the pool. A triangle, square, circle and two stripes were drawn on the flags to facilitate spatial orientation. Spatial learning was measured using a system consisting of an A Sony Color SSC-DC378 P camera and a workstation equipped with version 2.5 of the SMART software (PanLab, Spain).

The test was divided into two parts. The first part consisted of acquisition training (learning phase, day 1–5) while the second part included the trial session (test phase, day 6). The test was carried out according to the protocol reported by Bromley-Brits K. et al. [37]. On day 1, the animals were habituated to the swimming pool environment and the platform was placed 1 cm above the water level (visible platform). The length of time it took the animals to find the platform was recorded, and the visual perception of the animals was evaluated. On days 2–5, the platform was placed in a fixed position (south-west, SW) and 1 cm below the water level (hidden platform). The following parameters were analysed: the time taken to find the hidden platform, Whishaw's error (a measure of the mean orientation used to assess the time spent within the "corridor" connecting the starting point with the goal. If the animal was in the "corridor", there was a 100% error value) and the mean velocity.

In the learning phase (day 1–5), five trials were carried out every day. In those trials, the mice were placed in the pool for one minute in one of the four starting points (N, W, S, E). If the mice located the platform, they were allowed to remain on it for five seconds. If the animal failed to find the platform, it was directed toward the platform, and then it was placed on it for 20 s.

In the probe trial, the platform was removed and an N - drop location was established. The mice were placed in the pool individually for one minute, and the distance travelled and the time in the target zone (SW) were calculated.

2.5. Statistical analysis

Statistical analysis was carried out using Statistical R (version 3.0.1) and MedCalc (version 12.7.7) software. Repeated measures ANOVA was used for variables measured at more than two time points. A post hoc analysis of multiple comparisons with the Holm correction was carried out. A classical one way ANOVA was used to statistically analyse the results obtained on the test day in more than two groups without the platform (high dose, low dose and placebo group). A post hoc analysis of multiple comparisons with the Holm correction was also carried out. Student's *t*-test or Welch's *t*-test (depending on the results of the F-test) were used to statistically compare the results of two groups (placebo subgroups) on the test day. The normal distribution of data was assessed using the D'Agostino-Pearson test. No significant deviation from normality was observed. The results were statistically significant when the p-value equalled 0.05 or less.

2.6. Ethical issues

The experiment was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and was approved by the Ethical Committee on the Animal Research of the Institute of Immunology and Experimental Therapy Polish Academy of Sciences in Wroclaw, Poland (no. 58/2010; 2/2012; 8/2012; 9/2012; 27/2012).

3. Results

3.1. Body weight

The body weight of the animals in all the AD and NCAR groups increased significantly during the 24 weeks from the start of the study (AD: 24.89 ± 2.25 g; NCAR: 24.34 ± 1.60 g) until its completion (AD: 29.17 ± 2.55 g; NCAR: 30.11 ± 2.95 g.) (AD: $F_{5,134} = 24.72$, $p < 0.001$; NCAR: $F_{4,89} = 31.93$, $p < 0.001$). The increase in the body weight in the AD and NCAR groups receiving ovocystatin was similar to that in the corresponding vehiculum groups (AD: $F_{2,24} = 1.84$, $p = 0.180$; NCAR: $F_{2,22} = 0.50$, $p = 0.614$) (data not shown).

3.2. Locomotor activity

There was no statistically significant difference in slow movements between the groups (AD: $F_{2,24} = 0.068$, $p = 0.934$; NCAR: $F_{2,22} = 0.31$, $p = 0.739$). There was a significant decrease (AD: $F_{1,24} = 16.78$, $p < 0.001$; NCAR: $F_{1,22} = 25.80$, $p < 0.001$) in motor activity in all the groups after 12 weeks of administering ovocystatin or the vehicle substance, which persisted until the 22nd week, when the MWM test was performed (Fig. 2).

3.3. Morris water maze – spatial learning test

3.3.1. Acquisition training

There was no difference in the latency to target between the AD and NCAR vehiculum groups in the test with the visible platform (AD (vehiculum) vs. NCAR (vehiculum), $p = 0.065$). Ovocystatin administered in both doses did not affect the latency to target in the AD mice ($F_{2,24} = 0.358$, $p = 0.703$). Wild-type mice that received ovocystatin (NCAR + CYS4) reached the visible platform faster than the mice from the NCAR (vehiculum) group and those from the NCAR + CYS40 group (latency to target 33.31 ± 6.80 s vs. 38.83 ± 7.48 s and 33.31 ± 6.80 s vs. 42.41 ± 7.28 s, $F_{2,22} = 3.481$, $p < 0.05$, respectively) (Fig. 3a).

During the 2–5 days of acquisition training, all the AD ($F_{3,72} = 14.82$, $p < 0.001$) and NCAR ($F_{3,66} = 22.00$, $p < 0.001$) groups of mice demonstrated shorter latencies to find the hidden platform on the consecutive days. The AD (vehiculum) mice had significantly longer swimming latencies than the NCAR (vehiculum) mice on the 2nd, 3rd, 4th and 5th day of the acquisition trial ($F_{1,15} = 9.49$, $p = 0.008$). No significant differences between the groups treated with ovocystatin and the AD ($F_{2,24} = 2.29$, $p = 0.123$) and NCAR ($F_{2,22} = 0.20$, $p = 0.823$) vehiculum groups were noted (Fig. 3b).

In addition, the behavioural analysis showed that the mice swam more accurately from the starting point to the hidden platform on consecutive days (Wishaw's Error increased on each day of the acquisition learning) (AD: $F_{3,72} = 4.95$, $p = 0.004$; NCAR: $F_{2,48} = 11.40$, $p < 0.001$). However, there was no statistically significant difference in Wishaw's Error between the AD and the NCAR vehiculum mice ($F_{1,15} = 0.23$, $p = 0.642$). Similarly, the administration of ovocystatin did not increase the accuracy with which the AD and NCAR mice located the hidden platform (AD: $F_{2,24} = 0.46$, $p = 0.638$; NCAR: $F_{2,22} = 0.64$, $p = 0.536$) (Fig. 3c).

There was no statistically significant difference in the mean velocity among the groups of mice ($p > 0.05$) (data not shown).

3.3.2. Probe trial session

In the probe trial, memory performance was significantly impaired in the AD (vehiculum) group compared to the NCAR (vehiculum) group on the 6^{th} day, as shown by a decrease in the travelled distance (Fig. 4a), and prolonged permanence time (Fig. 4b) in the SW zone,

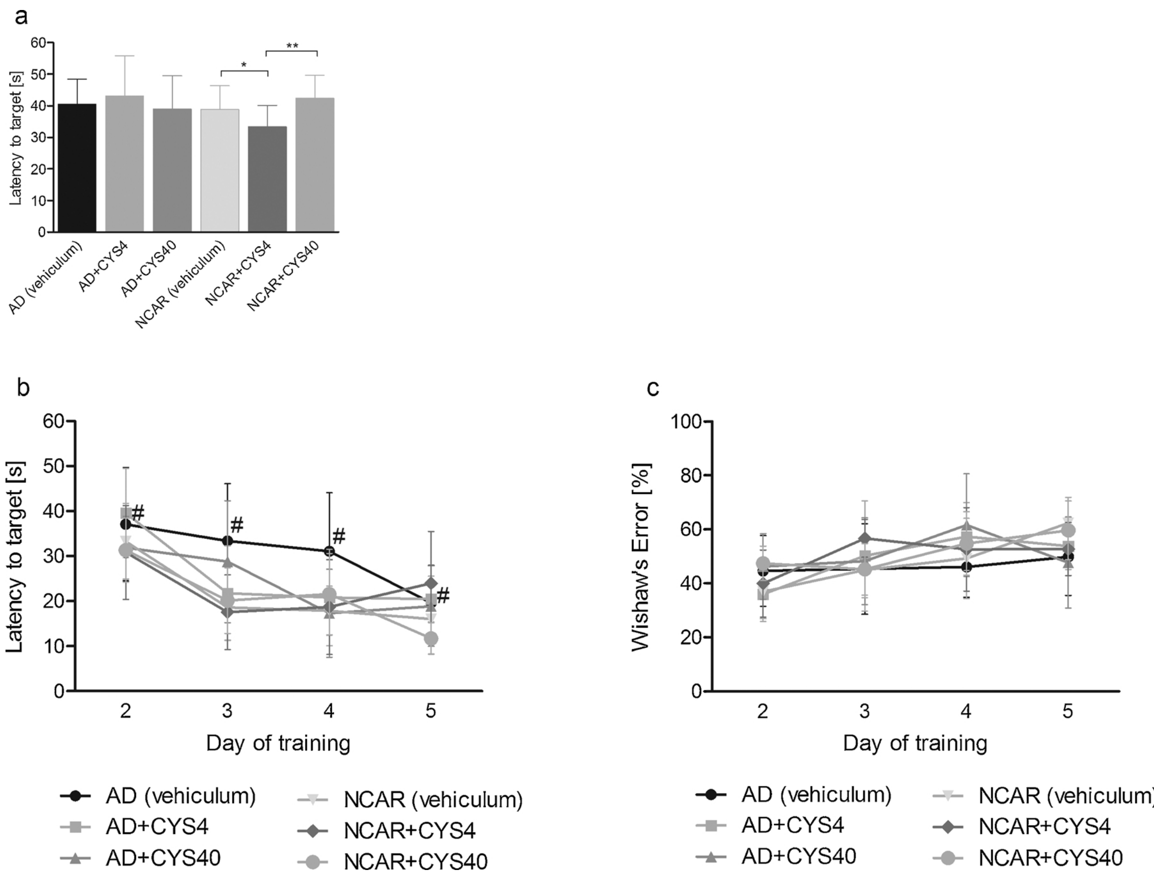

Fig. 3. Effects of ovocystatin on the acquisition training in the MWM test. Latency to find visible platform (day1) (a); latency to find hidden platform (b) and Wishaw's Error (c) during day 2–5. Data are expressed as mean ± SD *p < 0.05 NCAR + CYS4 compared with the NCAR (vehiculum), **p < 0.05 NCAR + CYS4 compared with NCAR + CYS40, # p < 0.05 AD (vehiculum) compared to NCAR (vehiculum).

where the platform was previously hidden (the target zone) (AD (vehiculum) vs. NCAR (vehiculum), $p < 0.05$). A higher dose of ovocystatin led to a significant increase in the distance travelled by the mice with AD (AD + CYS40 vs. AD (vehiculum): $F_{2,24} = 4.578$, $p = 0.021$). In the AD + CYS40 group, the permanence time in the target zone also increased, but the difference was not statistically significant (AD + CYS40 vs. AD (vehiculum): $F_{2,24} = 3.105$, $p = 0.063$). The administration of a lower dose ovocystatin also caused an increase in the studied parameters, but the differences were not statistically significant. Ovocystatin did not affect the travelled distance and permanence time for the NCAR mice.

4. Discussion

The study of the potential protective effect of ovocystatin against memory loss was carried out using a double-transgenic mouse model, which expressed the mouse/human amyloid precursor protein containing the K595N/M596L Swedish mutation and mutations in the presenilin 1 deltaE9 gene, which is an exon-9 deletion responsible for early onset Alzheimer's disease [35,36]. The mice were tested in the MWM, which is used to evaluate learning ability and spatial short and long-term memory [38,39].

Our results show a significant decrease in the distance travelled in the target zone by AD mice compared to the NCAR mice in the probe MWM trial. This indicates that AD mice had impaired spatial memory. Long-term administration of ovocystatin at 40 µg/mouse reduced memory impairment caused by an APP overexpression and extended the distance travelled in the target zone. Due to the fact that ovocystatin did not affect the physical activity of the mice in the actimeter test, its beneficial effect in the MWM was not caused by improved motor activity.

To the authors' best knowledge, this is the first study assessing the effect of ovocystatin on the cognitive function in APP/PS1 transgenic mice. The obtained results suggest that ovocystatin may have protective effects and may reduce memory deficits in Alzheimer's disease. They confirm that cystatin plays an important role in the development of Alzheimer's disease. Moreover, it is consistent with our earlier study [27], which indicates that ovocystatin administered orally to young rats may significantly improve memory in the MWM test. Similarly, we also revealed that ovocystatin delivered intraperitoneally may prevent age-related cognitive decline, but the obtained results were not statistically significant [28]. In addition, these results are in accordance with the study by Hook et al. [40], who found that cysteine protease inhibitors significantly reduce cognitive deficits in London APP mice. Apart from an improvement in the animals' memory tested with the MWM, Hook et al. [40] noted a reduction of amyloid plaques and a decrease in the β-secretase activity in those mice.

Cystatin C can bind to Aβ and inhibit its oligomerization and amyloidogenesis [13,41]. Most of the research conducted on transgenic mouse lines points to an anti-amyloidogenic and neuroprotective role of cystatin C. Steinhoff et al. [42] found that mice with APP_{swe} expression had increased levels of cystatin C in the brain. The expression of human cystatin C in the mice with a mutation in the APP gene caused a reduction in the Aβ aggregation in the brain through binding of cystatin C to Aβ [18,43]. Ghidoni et al. [44] reported that two mutations (PS2 M239I and T122R) in the presenilin 2 gene associated with familial Alzheimer's disease resulted in decreased extracellular levels of cystatin, which may impair neuroregeneration. However, another study found that cystatin removal may have a protective effect. Genetic ablation of the *CST3* gene encoding cystatin decreased the level of soluble

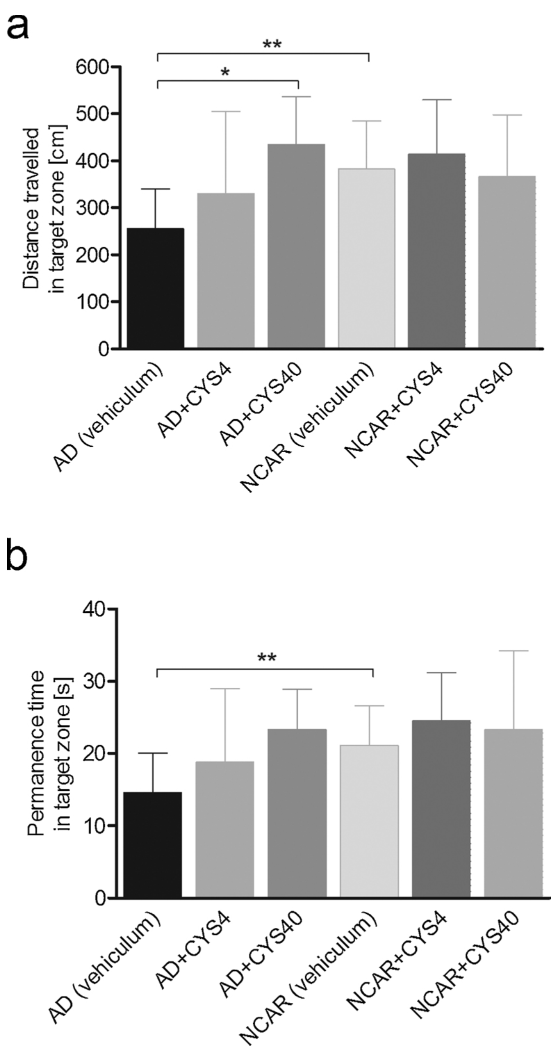

Fig. 4. Detailed results of the MWM probe trial session. All results are mean ± SD. Data are expressed as the distance travelled (a) and permanence time (b) in the SW target zone. * indicates a significant difference compared to the AD (vehiculum) group ($p < 0.05$); ** indicates significant difference between the AD (vehiculum) and NCAR (vehiculum) mice ($p < 0.05$).

Aβ and diminished cognitive deficits in the MWM [45].

One important protective mechanism of cystatin C is the inhibition of cysteine proteases. The concentration of cysteine proteases increases in response to brain tissue injury. Modified proteolysis caused by an imbalance between active proteases and endogenous inhibitors may occur physiologically during aging or pathologically in numerous neurological disorders, including Alzheimer's disease [46]. The inhibitory function of cystatin C is further confirmed in cystatin C knockout mice, which have an elevated cathepsin B activity [45]. Other studies have found that cystatin C and the fibroblast growth factor 2 (FGF-2) may stimulate neurogenesis [47], and that cystatin C secretion induces the proliferation of neural stem/progenitor cells (NSCP), stimulated by the expression of APP [48]. Furthermore, cystatin C plays a neuroprotective role through inducing autophagy, which increases the degradation of long-lived proteins [49]. Similarly, Watanabe et al. [50] found that cystatin C exhibits neuroprotective effects by inducing autophagy and inhibiting cathepsin B.

Clinical studies also imply the protective effects of cystatin C in Alzheimer's disease. Patients with Alzheimer's disease have low cystatin levels. An analysis of serum cystatin C levels revealed that a decrease in cystatin C precedes the clinical manifestation of Alzheimer's disease [51], and may incite the conversion of mild cognitive impairment into Alzheimer's disease [52].

To date, few in vivo studies on the activity of exogenous cystatin have been carried out. In the study by Nagai et al. [53], cystatin C was administered directly into the rat hippocampus. Despite neuronal damage in the dentate gyrus, the immunohistochemical analysis revealed that Aβ was not deposited in the hippocampus following the cystatin C administration. That finding supports the hypothesis that exogenous ovocystatin inhibits Aβ oligomerization and deposition, which may reduce cognitive deterioration and improve cognitive functions. Liu et al. [54] found that the administration of cystatin C in a rat model of subarachnoid haemorrhage decreases cognitive deficits, which is in accordance with the findings of the present study. However, Liu et al. [54] found an enhancement of learning abilities in the probe MWM trial, while the authors of the current study found memory improvement in the same type of trial.

This study reports an innovative method of long-term administration of ovocystatin in drinking water, as well as its use as a protective and anti-dementia agent. The survival of the mice during the 24 weeks study period suggests that ovocystatin is safe to use. Toxicological studies are warranted to confirm this hypothesis. At this stage the authors cannot delineate the molecular mechanisms, in which orally administered ovocystatin exerts its protective effects. It is known that some proteins, including a close homolog of cystatin C – cystatin S – can, to some extent, pass the jejunal epithelium and reach vascular compartments [55–59]. The passage of certain proteins through the blood-brain barrier has also been reported. For example, 0.1% of circulating antibodies can reach the brain at a steady-state concentration [60]. Generally, few pathways are believed to facilitate protein molecule transport across the blood-brain barrier, and they include receptor-mediated transcytosis, adsorptive endocytosis and paracellular transport. The passage of cystatin C through this barrier is apparent as 1% of the protein content in the cerebrospinal fluid is derived from circulation [61]. Thus, it is conceivable that in the current model, the administered ovocystatin reached the brain tissue and directly acted on the injured area by way of its anti-aggregative and anti-inflammatory properties. Alternatively, the effect of ovocystatin may result from its activity outside the brain. There is compelling evidence that apart from CNS inflammation, the peripheral immune response may contribute to the development of neurodegenerative diseases. Cytokine mediated regulation of inflammatory signals in the systemic circulation and along the microbiota-gut-brain axis have been linked to the pathogenesis of Alzheimer's disease [62,63]. On the other hand, several studies indicate profound immunomodulatory and antimicrobial activities of cystatins, including ovocystatin [64,65]. Due to systemic modulation of the immune system or the alteration of the composition of the gut microbiota and certain metabolic interactions, ovocystatin may exert its effect via circulation or via the gastrointestinal tract gut. Further studies are necessary to evaluate the pharmacokinetics, the stability, the distribution and the mode of action of this protein in the human body.

In the recent years, there has been growth in the development of large scale methods of egg white protein preparation. It can soon be expected that this could lower the future cost of egg protein preparation for the use in nutraceutics [66].

The obtained results encourage future studies into the use of ovocystatin in Alzheimer's disease. At the same time, the study had some limitations. The proposed model only included FAD, a rare form of Alzheimer's disease. In addition, an animal model was used to assess the action of ovocystatin, which may have different mechanisms of action in humans. Furthermore, the study was conducted only on male mice. Thus, the authors were unable to assess the influence of gender on the cognitive behaviour of mice after administering ovocystatin. The dose of ovocystatin was chosen based on the authors' preliminary data (unpublished data). Further studies using different dosages of ovocystatin are warranted. Ovocystatin acquisition is costly and in order to produce ovocystatin on a large scale, more efficient manufacturing methods are needed [67]. This study only assessed the effect of ovocystatin on cognitive functions. The authors have not evaluated the effect of oral administration of ovocystatin on the expression of endogenous mouse cystatin C. However, it is known that the inhibitor belongs to a group of housekeeping proteins that are normally synthetized at a constant level across the entire life-span [11]. Further morphological, biochemical and immunohistochemical studies should be carried out on the animal models.

The authors found that ovocystatin has biological properties that may inhibit the deterioration of cognitive functions. Further research is needed to confirm the effect of ovocystatin on aging and on Alzheimer's disease.

5. Conclusions

In conclusion, our results suggest that ovocystatin may reduce memory impairment in the course of Alzheimer's disease. In contrast to other studies suggesting a positive correlation between the concentration of cystatin and cognitive deficits, the authors of this study did not find any effect of cystatin on memory and learning in wild-type mice. Ovocystatin appears safe in long-term use. Nevertheless, further studies are needed to determine whether it can be used in the adjuvant treatment of Alzheimer's disease.

Conflict of interests

The authors declare no conflict of interests.

Financial disclosure

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The authors contribution

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Statistical Analysis: Bartłomiej Stańczykiewicz,

Data Interpretation: Bartłomiej Stańczykiewicz.

Manuscript Preparation: Bartłomiej Stańczykiewicz, Maria Rutkowska, Jakub Gburek, Krzysztof Gołąb, Joanna Rymaszewska.

Literature Search: Bartłomiej Stańczykiewicz, Maria Rutkowska, Jakub Gburek, Krzysztof Gołąb.

Funds Collection: Joanna Rymaszewska, Antoni Polanowski, Jakub Gburek, Tadeusz Trziszka.

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