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Potential protective effect of ovocystatin on aging-related cognitive impairment in rats

Wpływ ovocystatyny na funkcje poznawcze szczurów*

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: Increased occurrence of cognitive deficits in mild cognitive impairment is related with the phenomenon of aging within the population. Cystatin C has been associated with cysteine protease inhibiting properties as well as an induction of autophagy and proliferation that can potentially be used as an adjuvant in the treatment of cognitive decline. The aim of the study was to evaluate the effect of ovocystatin, which is structurally and biologically similar to cystatin C, on cognitive functions in experimental young and aging rat models.

Material/Methods: The young (four-month-old) and aging (ten-month-old) Wistar Crl: Wi (Han) rats received ovocystatin (i.p.) for 12 days at a dose of 200 and 20 μg/rat, respectively. Cognitive functions were determined using the Morris water maze.

Results: Ovocystatin treatment at a dose of 200 μg/rat improved the performance of old rats in the Morris water maze test via increasing the spent time and the distance traveled in the target zone but the differences were not statistically significant ($p>0.05$). The results of the study highlight the important role cystatins play in neurodegenerative processes as well as the influence they have on cognitive functions. Furthermore, the obtained findings suggest ovocystatin may be used in the treatment of mild cognitive impairment or cognitive decline in dementia, but further morphological, biochemical and immunohistochemical studies are needed.

Keywords: **ovocystatin • chicken egg white • cystatin C • Morris water maze • cognitive decline • rat**

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Introduction

Normal cognitive aging is mild and affects memory, psychomotor speed and spatial orientation. Aging of the brain affects mechanisms responsible for the regeneration of neurons damaged by free radicals or inflammation. It also alters brain metabolism and causes a decrease in neurogenesis and angiogenesis. This, in turn, leads to central nervous system deterioration, influencing learning and memory [17]. Aging also causes altered proteolysis, which results from an imbalance between proteases and their endogenous inhibitors [9]. Early detection of the risk factors for cognitive impairment makes it possible to seek new therapeutic methods and facilitate healthy aging [5]. Over the past 20 years, there have been genetic, experimental and clinical studies suggesting a relationship between cystatin C (CysC) and cognitive functions [45]. CysC is a type 2 endogenous cysteine protease inhibitor. In humans, it was first identified in the cerebrospinal fluid [24]. It was then found to be present in all mammalian tissues and body fluids. Cystatin is highly abundant in brain tissue (in neurons, endothelial and glial cells, including astrocytes) and is five-fold more concentrated in the cerebrospinal fluid than in the peripheral blood. This suggests its important role in the central nervous system (CNS) [35]. The secretion and expression of CysC are altered in various neurological disorders and animal models of neurodegenerative diseases. This indicates that CysC plays an important role in the course of these diseases [7]. Numerous *in vitro* and *in vivo* studies found that CysC has neuroprotective effects and acts by inhibiting the activity of cysteine proteases (such as cathepsin B), inducing autophagy and intensifying cell proliferation in acute and chronic neurodegenerative disease [4,25,28]. Additionally, potential therapeutic properties of CysC in Alzheimer's Disease have been suggested. Namely, Wang et al. [42] revealed that CysC reduces Aβ40 secretion in brain endothelial cells. Nevertheless, clinical studies assessing the relationship between CysC and cognition have produced conflicting results. Two studies in longitudinal cohorts of elderly men and women showed that a higher level of CysC is associated with poor cognitive functions and an increased risk of cognitive decline [29,45]. Similar results were found in elderly patients with chronic kidney disease [19,44] and in patients with Parkinson's disease [20]. In contrast, a long-term study carried out on a large group of patients found that low serum CysC levels precede the clinical manifestation of Alzheimer's disease. It was discovered that the increased risk of developing the disease was independent of age, the apolipoprotein E (ApoE4) genotype, the glomerular filtration rate, the body mass index, concurrent diseases or smoking [35]. The results obtained in another studies also suggest low plasma CysC levels in the disease [8]. According to Slinin et al. [32], there may be a U-shaped association between the CysC concentration and the risk of cognitive impairment or dementia. Those authors found that both low and high CysC levels may increase the risk of cognitive decline in elderly patients, and that this relationship was not independent of confounding factors. There are also studies that did not find any relationship between CysC and cognition. Alosco et al. [1] found that in patients following bariatric surgery, low CysC levels were not associated with improved cognitive function. To date, there has only been one study assessing the effect of exogenous CysC on cognitive function. In a rat experimental model of subarachnoid haemorrhage (SAH), CysC infused into the prechiasmatic cistern diminished learning deficits caused by brain damage [18].

Our study assessed ovocystatin (ovCys), which is the best-characterised type 2 cystatin protein. Ovocystatin is used in a variety of experimental studies as a model protein and is considered representative of this cystatin superfamily [11,24]. Ovocystatin is a C1 inhibitor of cysteine peptidases, such as cathepsin B, H, K, L and S [9,11] and shares biological properties with human CysC. The aminoacid sequence of ovocystatin is 44% homologous to human cystatin C, and the two proteins share 62-63% of structure. The secondary crystal and solution structures are also similar [14,24,43]. Unlike human CysC, ovocystatin is easily accessible as it is obtained in large amounts from chicken eggs.

Ovocystatin, as well as other cystatins belonging to cystatin family-2, have been shown to exert several interesting biological properties, such as anticancer, antimicrobial, proapoptotic and immunomodulatory activities. Therefore, the use of cystatins as an active agent of drugs in therapy of cancer, inflammation and bacterial infections is seriously considered, in addition to their possible use in treatment of neurodegenerative disorders [15,21,22,31,36,41]. Taking into account the

suggested protective role of CysC in neurodegenerative diseases [23] as well as the structural and biological properties' similarity between CysC and ovocystatin, the present study was carried out to investigate the effect of ovocystatin on aging-related cognitive impairment in rats.

Material and Methods

Ethical statement

The study 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 of the Polish Academy of Sciences in Wroclaw.

Isolation and characterization of ovocystatin

Ovocystatin was prepared using affinity chromatography on Cm-papain-Sepharose, according to the method described by Anastasi and co-workers [2] with later modifications [22]. The efficiency of the preparation was calculated on the basis of inhibitory activity against papain and the protein concentration in an egg white homogenate and the final product, respectively. The anti-papain activity of the inhibitor was measured colorimetrically against α-N-Benzoyl-DL-arginine β-naphthylamide (BANA) as a substrate. One unit of the enzyme activity hydrolyzed 1μmol of the substrate/min under reaction conditions. The anti-papain activity of ovocystatin was measured after preincubation with the enzyme for 5 min at 40°C before adding the substrate. One inhibitor units corresponds to the amount that decreases the papain activity by one enzyme unit [3,40]. The protein concentration was determined using extinction coefficient at 280 nm $A^{0.1\%}$ = 0.871. The purity of ovocystatin was checked by SDS-PAGE in 12% gel under reducing conditions (Fig. 1) [30]. Based on electrophoresis, the inhibitor was pure and not aggregated. The specific antipapain activity of the purified inhibitor ranged from 20-25 U/mg protein. The purified ovocystatin was lyophilized as described by Gołąb and co-workers [9].

Animals, administration and experimental procedures

Four month-old (250-300g, n=24) and ten month-old (450–460g, n=27) male Wistar Crl:WI rats, purchased from the Experimental Medicine Centre of Medical University of Bialystok, were used. The rats were housed in cages in pairs under standard laboratory conditions (temp. 21±2°C, 12 h light-dark cycle with lights on at 7:00 A.M.). Tap water and food pellets were provided ad libitum.

The young (Y) and aged (A) rats were divided into six groups: 1. Y (vehicle, n=8), 2. Y+ovCys200 (200μg/rat, n=8), 3. Y+ovCys20 (20μg/rat, n=8), 4. A (vehicle, n=9), 5. A+ovCys200 (200μg/rat, n=9), 6. A+ovCys20 (20μg/rat, n=9). There was no statistically significant difference in the body mass of the aged rats and young rats receiving ovocystatin. A saline solution and ovocystatin were administered intraperitoneally at 1ml/rat once daily for 12 days.

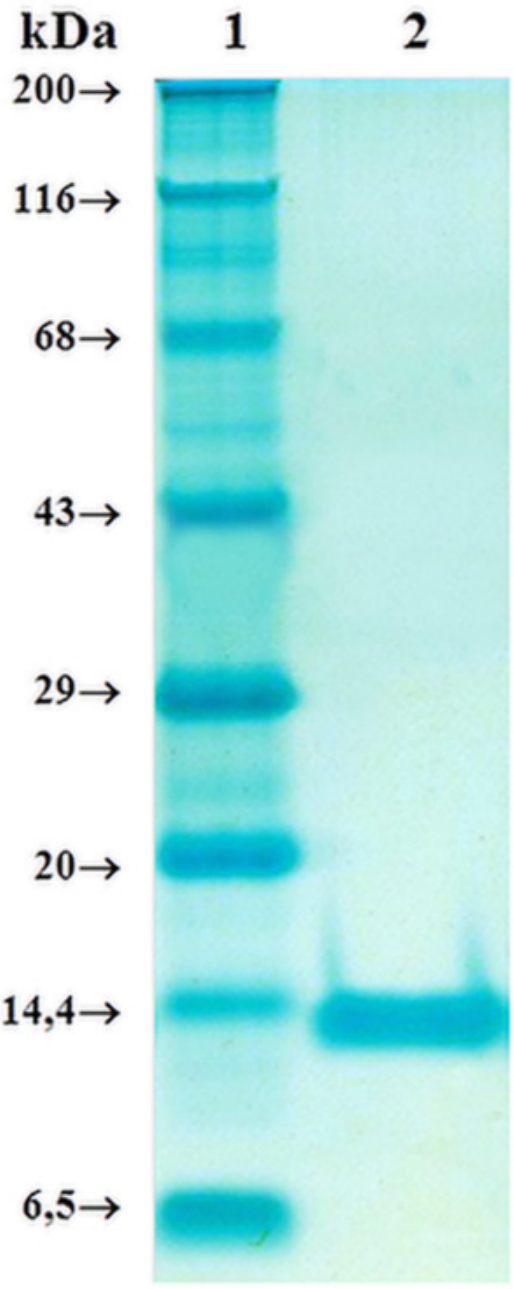

Fig. 1. Characterization of ovocystatin in SDS-PAGE. Lane 1. Molecular mass standards; Lane 2. Ovocystatin (2 μg). Molecular mass of ovocystatin is 13,100 Da

Morris water maze test (MWM)

A round pool 180 cm in diameter was filled with tap water up to approximately 29 cm and maintained at 22°C. The water was colored black using a dye safe for animals. Visual cues in the form of flags were erected

on two sides of the pool and on two curtains surrounding the pool. A triangle, circle, square and two parallel lines, respectively, were drawn on the flags to facilitate spatial orientation. The pool was divided into four quadrants (NE, NW, SE, SW). Spatial learning and memory were measured using SMART software, ver. 2.5 (Pan-Lab, Spain).

The MWM test was divided into two phases – an acquisition trial (learning phase, days 1-12) and a probe trial (test phase, day 13). The vehicle or ovocystatin (depending on the rat group) were administered during the acquisition trial (days 1-12). In the acquisition trial, carried out 30 minutes after the substances had been administered intraperitoneally, the rats were placed in the swimming pool facing the wall at one of the four starting points (N, W, S or E). The starting position was changed every day. Each rat was given two minutes to find the hidden platform submerged 1 cm below the surface of the water in the SQ quadrant. If the animal located the platform within the allotted time, it was removed from the pool five seconds later. If the rat failed to locate the platform, it was directed toward the platform and placed on it for 20 seconds. The latency to find the hidden platform and Wishaw's Error (a measure of the mean orientation used to measure the time each animal spent within the "corridor" connecting the starting point with the goal) were recorded. If the animal swam in the "corridor", there was a 100% error value. The probe trial was performed after the last day of the learning phase (day 13). The hidden platform was removed and the rats were placed in the N-drop location individually for one minute. The results, including the percentage of the distance travelled and the time the rats spent in the target quadrant, were calculated for each animal.

Statistical analysis

R software for statistical analysis (version 3.0.1) and MedCalc (version 12.7.7) were used. The repeated measures ANOVA was used to compare variables that were measured at multiple time points, while the Holm correction was used to carry out a post-hoc analysis of multiple comparisons. A standard one-way ANOVA was used to compare the results of the test without the platform between several groups (depending on the dose and substance). Student's t-test or Welch's t-test (depending on the results of the F-test) were applied to compare the results of the test in the two placebo subgroups. The normality of the data was assessed using the D'Agostino-Pearson test. The results were statistically significant at $\alpha=0.05$.

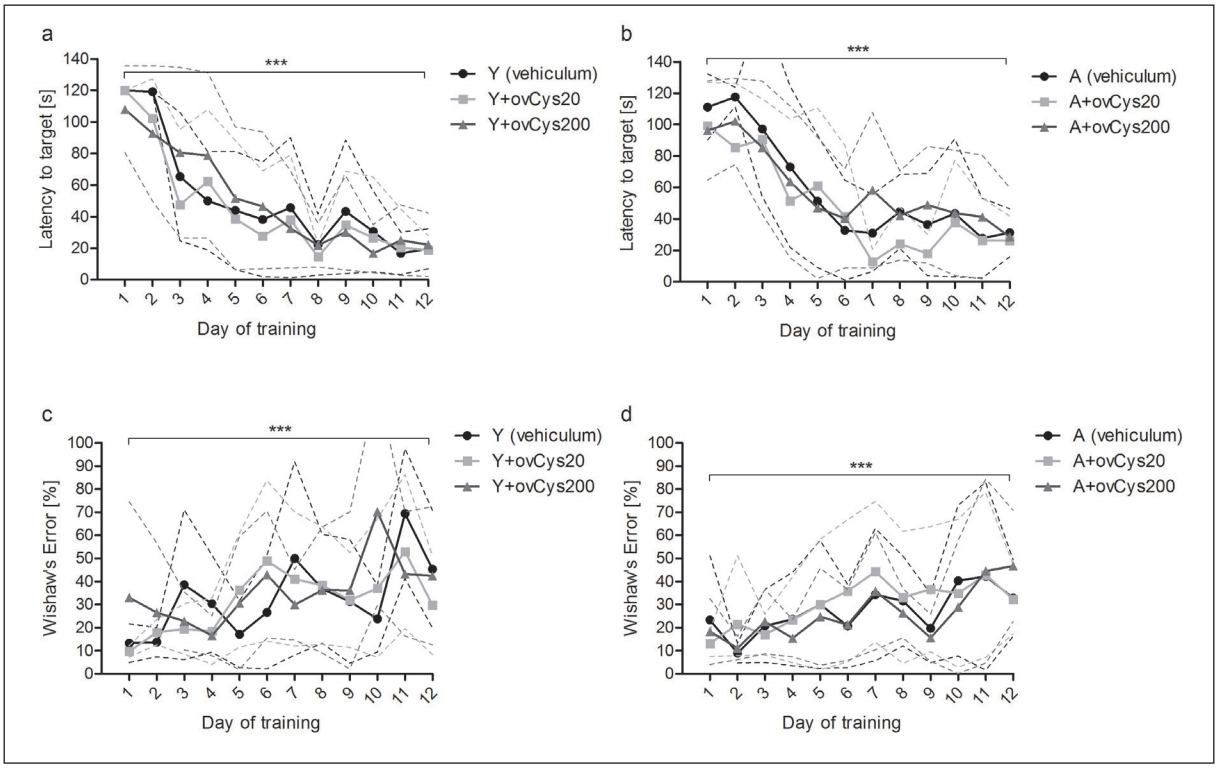

Fig. 2. The effect of ovocystatin on the latency to find the hidden platform (a, b) and accuracy in reaching the platform [Wishaw's error] (c, d). Data are expressed as mean ± SEM (for better legibility, SEM values are presented in dashed lines). ***p<0.001: a significant difference between first and last day of the acquisition phase. No statistical difference between groups treated with Ovocystatin and control group was found

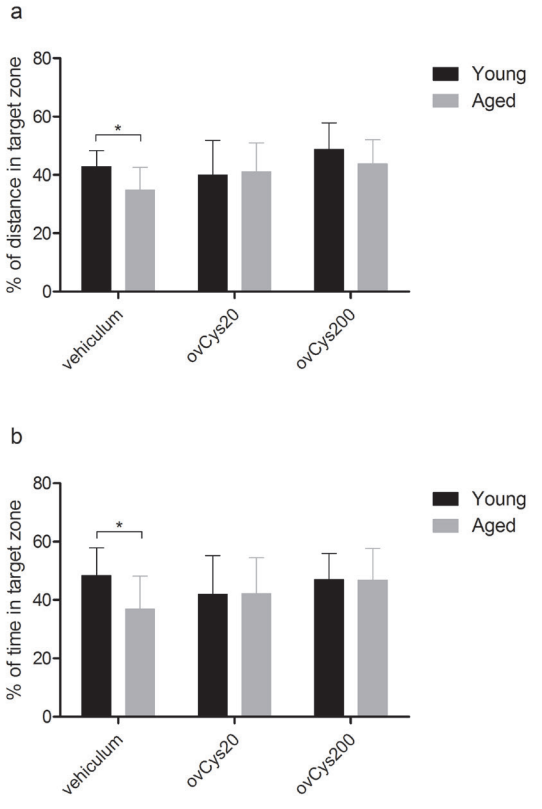

Fig. 3. Distance travelled (a) and time spent in target zone (b) as the % of total test trial distance and time, respectively. *- p<0.05 a significant difference between groups of young and aged rats treated with saline. Data are expressed as mean ± SEM

Results

Effects of ovocystatin in the MWM

In the training phase, there were no statistically significant differences between the groups in the latency to find the hidden platform (Fig. 2a, 2b) and in Wishaw's Error (Fig. 2c, 2d) (p>0.05). Over time, the latency decreased in all the groups of rats (p<0.001 compared to baseline values) and the precision of swimming to the goal platform (Wishaw's Error) increased (p<0.001 compared to baseline values), which indicates that all the animals learned to find the platform.

As shown in Fig. 3, aged rats spent significantly less time in the target zone than the young rats (Aged vs Young, p<0.05) (Fig. 3a) and traveled a shorter distance in the SW (target zone) (Aged vs Young, p=0.028) (Fig. 3b). In the aged rats, ovocystatin increased the time spent and the distance traveled in the target zone in a dose dependent manner. The obtained results were not statistically significant compared with the results of the aged and young rats treated with saline (p>0.05). In the young rats, ovocystatin administered at the two doses did not significantly affect the time spent and distance travelled in the target zone (p>0.05).

Discussion

The Morris Water Maze is a classic test for examining spatial learning and memory and is often used to assess cognitive deficits associated with aging. Mice and rats are used in this test as animal models of age-related cognitive deficits and to study the procognitive properties of certain drugs. It is believed that the development of cognitive deficits in rodents is associated with physiological aging rather than the death of brain cells [6].

In our study, we observed disorders of spatial memory in 10-month-old rats, as indicated by a significant decrease in the distance travelled and time spent in the target zone in the probe trial of the MWM compared to the 4 month-old rats. Ovocystatin administered to aged rats at two doses (20 μg/rat and 200 μg/rat) improved their memory, extending the distance travelled and time spent in the target zone. Even though the differences in the examined parameters were not statistically significant compared with the group of aged rats treated with saline, the results were similar to those obtained in the control group of young rats. Ovocystatin did not significantly affect learning in the training phase in either the young or the aged rats.

To the authors' best knowledge, this is the first study to assess the effect of ovocystatin on the cognitive function. The results suggest that ovocystatin has a protective effect on age-related cognitive impairment in rats. Our results are in accordance with previous studies [18], which showed a neuroprotective effect of CysC in a rat experimental model of SAH. In that model using the MWM, the authors found that administering CysC decreased the cognitive deficits caused by brain damage. At the same time, contrary to our findings, CysC improved learning but did not affect memory. It was assumed that CysC might have a protective effect on SAH by inducing autophagy. Autophagy may protect cells from apoptosis by eliminating damaged mitochondria or other organelles that may drive cells to apoptosis [39]. It was found that cystatin C induces autophagy by inhibiting the activation of the mTOR kinase (mammalian target of rapamycin), which is a negative regulator of autophagy.

In animal models of experimental transient ischemia, it has been shown that CysC has a protective effect by inhibiting the activation of proteases that are released in response to brain tissue damage. The inhibitory function of CysC is confirmed in CysC knockout mice, which have an increased activity of cathepsin B [33]. Studies on CysC knockout mice also indicate that CysC may reduce traumatic brain injury, although this effect is limited to certain areas of the brain [26]. CycC may also regulate cell proliferation [34,38]. It was found that the release of

CysC mediates the proliferation of neuronal neural stem/progenitor cells (NSPC), stimulated by the expression of the amyloid precursor protein (APP) [12]. Glycosylated CysC interacts with the fibroblast growth factor 2 (FGF-2) in the stimulation of neurogenesis in the dentate gyrus of the hippocampus of adult rats [37]. Decreased basic levels of cystatin C in the subgranular zone in the dentate gyrus of the hippocampus in CysC knockout mice also indicate that it plays a role in neurogenesis [27]. In addition, cystatin C may be involved in astrocyte differentiation during mouse brain development [16]. It could also affect the differentiation of embryonic stem cells into neural stem cells [13]. The effect of cystatin C on the development of glial cells, including astrocytes, was confirmed in the study by Hasegawa and co-workers [10].

The obtained findings encourage further studies concerning the effect of exogenously applied ovocystatin on cognitive decline or on other neurodegenerative related diseases. The present study has several limitations. The used animal model presents cognitive decline, mainly linked to the aging process. Thus, the studies using animal models of Alzheimer's Disease may be useful to establish not only influence on cognitive functions, but also possible mechanisms of action. Additionally, further studies examining different dosages of ovocystatin are needed. In the present study, the doses of ovocystatin were based on unpublished preliminary data. Thus, the morphological, biochemical and immunohistochemical research, including larger groups of animals and comparison with other procognitive or antidementia preparations, are warranted.

Conclusions

In conclusion, the results of our study suggest that ovocystatin may prevent age-related cognitive decline, though the obtained differences were not statistically significant. Therefore, ovocystatin seems suitable for nutritional adjuvant treatment of age-dependent cognitive impairment. However, further studies are needed to confirm the procognitive properties of ovocystatin and to establish its mechanisms of action.

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