

Cystatins: unravelling the biological implications for neuroprotection

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Abstract

Cystatins, a family of proteins known for their inhibitory role against cysteine proteases, have garnered significant attention in the field of neurodegeneration. Numerous genetic, experimental, and clinical studies concerning cystatin C suggest it plays an important role in the course of neurodegenerative diseases. Its beneficial effects are associated with cysteine protease inhibition, impact on β -amyloid aggregation, as well as regulation of cell proliferation, autophagy, and apoptosis. Cystatin isolated from chicken egg white, called ovocystatin, has been widely used in medical and pharmaceutical research due to its structural and biological similarities to human cystatin C. This article focuses on the potential use of cystatins, with special emphasis on easily obtained ovocystatin, in the treatment of neurodegenerative diseases, such as dementia. The current evidence on cystatin use has shed light on its mechanisms of action and therapeutic implications for neuroprotection and maintenance of cognitive functions.

Key words: ovocystatin, chicken egg white, cystatin C, neurodegeneration, neuroprotection.

Introduction

Alzheimer's disease (AD) is one of the most widespread and the most comprehensively studied neurodegenerative disorders. AD pathogenesis has been mainly attributed to extracellular aggregates of amyloid β ($A\beta$) plaques and intracellular neurofibrillary tangles (NFTs) made of hyperphosphorylated τ -protein in cortical and limbic parts of the human brain [1].

Mutations in the genes encoding amyloid precursor protein (APP) and presenilin 1 and 2 (presenilin 1 gene, PSEN1; presenilin 2 gene, PSEN2) are associated with Alzheimer's disease and lead to an increase in the production of toxic $A\beta$ [2, 3]. Additionally, the mutations in the PSEN1 and PSEN2 genes increase the production of an amyloidogenic form of $A\beta$ such as $A\beta_{42}$ [2]. It was also shown that the absence of presenilin expression in the neurons resulted in decreased NMDA receptor levels in the synaptic membrane and altered *CREB/CBP* gene regulation in the neuron, resulting in impaired BDNF expression [4].

Neurofibrillary tangles are another major pathological feature of AD. Under pathological conditions, abnormal phosphorylation of intracellular tau protein leads to the loss of its biological activity and causes microtubule depolymerization, which leads to degeneration of neurons [1]. Other proteins, such as apolipoprotein E (APOE), also play an important role. It was shown that the C-terminal fragments of protein APOE also can bind to A β , causing the growth of amyloid deposits. The ϵ 2 (APOE ϵ 2) and ϵ 3 (APOE ϵ 3) isoforms are mainly involved in the repair of neurons in the peripheral and central nervous system, and, in addition, play a major role in regulating the metabolism and distribution of cholesterol in neuronal membranes. However, the ϵ 4 isoform (APOE ϵ 4) is associated with the intensification of amyloidogenic processes. The occurrence of 2 copies of the APOE ϵ 4 allele increases the risk of developing AD 16-fold [5].

The development of neurodegenerative disorders such as AD may also be influenced by age-related changes in innate immunity, which provides the first line of defence by recognizing pathogen-associated microbial patterns, inducing key costimulatory molecules and cytokines that activate the adaptive immune response [6].

Current pharmacotherapy used during Alzheimer's disease is symptomatic and is administered to slow the disease progression and reduce the severity of behavioural disorders. The treatment strategy involves the use of cholinesterase inhibitors (donepezil, rivastigmine) and an N-methyl-D-aspartate receptor (NMDA receptor) antagonist (memantine) [7, 8]. The disease is primarily characterized by memory loss and decline of other cognitive functions, involving those associated with abstract reasoning, language difficulties, agnosia, and disorientation, but also behavioural or personality disorders, which affect the quality of life of both the patient and their loved ones. The illness significantly impacts the patient's daily professional activities and social functioning.

Hence, it is a serious health problem for patients and their families as well as the healthcare system [7, 9]. Projections assume that in the face of a rapidly aging population, there will be 80 million people worldwide suffering from dementia by 2040 [10]. Thus, research on preventive and therapeutic measures is warranted. Previous research on therapeutic strategies has focused on the amyloid cascade hypothesis. However, there is new research into the therapeutic effect of neuroprotective, anti-inflammatory, antioxidant, immunotherapeutic, chelating, and amyloid binding agents as well as secretase modulators [7]. This study presents the relationship between cystatin C (acquired from egg white) and neurodegenerative diseases, indicating its potential use in supporting cognitive functions.

Classification of cystatins

In 1981, Alan J. Barrett first used the term "cystatin" to describe a protein isolated from egg white that could inhibit the activity of lysosomal cysteine proteases [11]. The cystatins are a group of proteins typically composed of 100–120 amino acids [12]. They are papain-like cysteine protease inhibitors (C1) and some of them, like ovocystatin, also inhibit the Leguminosae family (C13) [11, 13].

Cystatin superfamily

The cystatin superfamily is divided into 4 types/families based on their characteristics as well as their presence in the intracellular, extracellular, and intravascular compartments (Figure 1). Type 1 cystatins, also named stefins, are mainly intracellular, unsynthesized cystatins that are pre-proteins containing signal peptides. Proteins representing this family are named "human stefin", "human cystatin A", "rat cystatin", "human cystatin B", and "rat cystatin B". They contain about 100 amino acid residues (~11 kDa) and lack disulphide bonds and carbohydrate side chains [14]. Stefin B has been shown to have an anti-inflammatory character via regulation of caspases and NF- κ B activation, and also to regulate lysosomal proteolytic activity and therefore intracellular clearance [15, 16]. Type 2 mainly contains cystatins secreted extracellularly, such as cystatins C, D, E, E/M, F, S, SN, and SA. They possess about 120 amino acid residues (~14 kDa) and 2 conserved disulphide bonds at the carboxyl terminus. Type 3 cystatins, also known as kininogens, include low- (50–68 kDa) and high- (88–114 kDa) molecular-weight intravascular cystatins, which are mainly plasma proteins and contain 3 cystatin domains [11, 13, 17]. They are synthesized in the liver in the form of multidomain glycoproteins [18]. It was also shown that kininogens are involved in

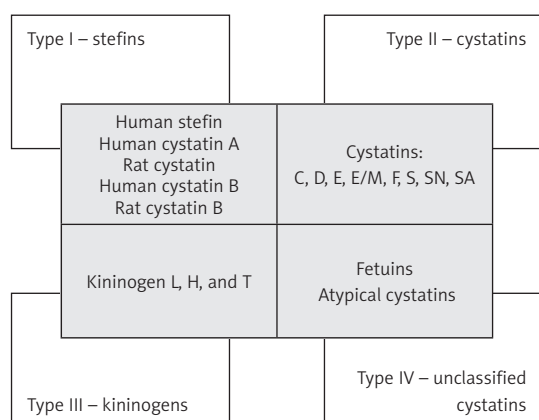


Figure 1. Cystatin superfamily

both innate and adaptive immune responses [19]. The type 4 group, containing fetuins and unclassified “atypical” cystatins, is distinguished in some classifications [11, 20, 21]. Another classification system is based on the sequence similarity and 3D spatial structure of the proteins. In this classification, the cystatins belong to the I25 family, with a further division into 3 subfamilies: I25A, I25B (where ovocystatin was classified), and I25C [22] (Figure 1).

Cystatin C

The first studies on cystatin C date back to 1961, when Jorgen Clausen first described the presence of a protein in the cerebrospinal fluid, which he called γ -CSF (γ -cerebrospinal fluid) [11]. In the following years, its presence was reported in cerebrospinal fluid, urine, and abdominal and pleural effusions. Due to the γ -electrophoretic mobility, it was called the “ γ -trace” or “post- γ protein”. The final name “cystatin C” was established in 1984 by Alan J. Barret *et al.* [11, 20].

Structure and function of cystatin C

Cystatin C is an endogenous inhibitor of cysteine proteases and is classified as a type 2 cystatin. The active and mature form of human cystatin C is a non-glycosylated polypeptide chain with a molecular weight between 13.343 and 13.359 kDa. It is composed of 120 amino acids with 2 disulphide bonds: Cys 73-83 and Cys 97-117 [11, 20, 23–25]. It is monomeric both *in vivo* and *in vitro*. However, it may be converted into a dimer in elevated temperatures, at a low pH, and by using chemical denaturation [12]. Cystatin C also undergoes 3D domain swapping, which can lead to the formation of amyloid plaques [11, 12, 17]. Human cystatin C is encoded by the *CST3* gene, located on chromosome 20 (20.p11.2), consisting of 3 exons and 2 introns [23, 26]. Its presence has been confirmed in all mammalian tissues and fluids, including the cerebrospinal fluid, blood plasma, semen, urine, and saliva [24, 25, 27]. It is also expressed in neurons, astrocytes, microglial cells, and the choroid plexus. Its presence in brain tissue is eminent [28, 29], and its concentration in the cerebrospinal fluid (CSF) is 5 times higher than in the blood [30]. Cystatin C has primarily been studied in the context of renal dysfunction and as a biomarker of the glomerular filtration rate (GFR). However, it has a much wider biological role involving its antibacterial and antiviral properties, a role in bone resorption, tumour metastasis, modulation of the immune system, and proliferation and cell growth [25, 28]. Brain cystatin C levels increase following injury, such as ischaemia, axotomy, or surgery [29]. The blood levels of cystatin C may increase in the

course of aging, chronic nephritis, neoplasia, urinary tract infections, hypertension, cardiovascular diseases, rheumatoid arthritis, and glucocorticoid pharmacotherapy [27, 28, 31]. As an endogenous inhibitor of cysteine proteases, cystatin C inhibits cathepsins B, H, L, and S [29, 32]. An imbalance between the proteases and inhibitors may result in the development of pathological conditions favouring excessive proteolysis and leading to abnormal tissue morphology or neuronal cell death [23, 33, 34].

The cystatin C gene and neurodegenerative diseases

Since human cystatin C was found to coexist with β -amyloid depositions, multiple studies have focused on the relationship between the *CST3* gene and neurodegenerative diseases. Cerebral amyloid angiopathy (CAA) is characterized by the deposition of amyloid plaques in the vascular wall of arteries, arterioles, and, less commonly, capillaries and veins. The Icelandic type of CAA, known as hereditary cystatin C amyloid angiopathy (HCCAA) or hereditary cerebral haemorrhage with amyloidosis of Icelandic type (HCHWA-I), leads to a haemorrhagic stroke and to the development of dementia. HCCAA is caused by a point mutation in the *CST3* gene (Leu to Gln mutation at position 68, Leu68Gln, L68Q) [28, 35]. The cystatin C gene has 3 polymorphic sites in the 3' region. The G/A polymorphism (G73A) of the *CST3* gene in the +73 position leads to an alanine to threonine substitution in the region encoding the signal peptide. It should be mentioned that the polymorphism responsible for encoding alanine is known as allele A, while the one responsible for the expression of threonine in the same position is known as allele B [36–38]. The study by Finckh *et al.* [29], carried out on AD patients and controls ± 75 years old showed that haplotype B of the *CST3* gene is associated with late-onset Alzheimer's disease, regardless of *APOE* ϵ 4. Moreover, the study by Crawford *et al.* [39] suggests that the *CST3*-A gene may be a risk factor for late-onset Alzheimer's disease, also regardless of the *APOE* ϵ 4. However, Cathcart *et al.* [40] showed that the *CST3*-A and *APOE* ϵ 4A gene interaction led to a 14-fold increased risk of AD in men and a 16-fold increased risk in women. Additionally, Beyer *et al.* [41] suggested that the *CST3*-A gene may be a risk factor in early-onset Alzheimer's disease. Furthermore, a synergistic association between the *CST3*-A and *APOE* ϵ 4 gene, and AD was found in patients with Alzheimer's disease between 60 and 74 years old. Another cystatin C polymorphism (Ala25Thr) is associated with both late-onset Alzheimer's disease and macular degeneration and leads to alternative signal sequence cleavage. In consequence,

the altered cystatin C is prone to aggregate formation and loses the inhibitory function of the protein in relation to both β -amyloid and cysteine proteinases [42, 43].

However, some studies provide mixed findings about the association between these genes and AD. Namely, Maruyama *et al.* [44] did not find any association between the *CST3* gene polymorphism and Alzheimer's disease. A similar finding was reported in the Italian population, where there was no relationship between AD and *CST3* either alone or in conjunction with *APOE* ϵ 4 [45]. In addition, a clinical control study also showed no association between the *CST3* gene, age, *APOE* ϵ 4, and Alzheimer's disease [37]. Nevertheless, a study conducted on the Chinese population showed that, although the *CST3A* allele frequencies were comparable in AD and the control group, the *CST3A/A* homozygote was significantly associated with late-onset AD [46]. Other studies on the Chinese population revealed the associations between *CST3B/B* genotype and AD patients older than 75 years, or VD patients younger than 75 years. Moreover, both *CST3* and *APOE* ϵ 4 genes were shown to be risk factors for vascular dementia [47]. Research into the Finnish population, carried out by Helisalmi *et al.*, did not find a link between the *CST3* gene and the risk of Alzheimer's disease [48]. Considering the inconsistent results of studies in this field, including those mentioned above, Hua *et al.* carried out a meta-analysis of the relationship between the cystatin C G73A polymorphism and Alzheimer's disease. The analyses indicate that this association is present in the Caucasian population and is absent in the Asian population. However, it has to be mentioned that the overall studied Asian population was smaller than the Caucasian one [49]. Babiloni *et al.* revealed the relationship between AD genetic risk factor and electroencephalography results in which the alpha 1 and alpha 2 amplitudes were statistically larger in patients with the B haplotype of the *CST3* gene than controls [36]. Maetzler *et al.* showed that the BB genotype of the *CST3* gene is associated with low cystatin C levels in the cerebrospinal fluid in patients suffering from Lewy body dementia [50]. Furthermore, the relationship between cystatin C and presenilin 2 was also found. Namely, 2 mutations of the presenilin 2 gene (PS2 M239I and T122R), linked to familial Alzheimer's disease, result in a reduction of extracellular secretion of cystatin C [51].

Cystatin C as a biomarker

Previous studies have attempted to determine the relationship between cystatin C levels in cerebrospinal fluid and blood and cognitive impairment or Alzheimer's disease. The level of cystatin C

in the cerebrospinal fluid was found to decrease in patients with AD [52]. On the other hand, higher CSF cystatin C concentrations correlated with more aggravated hippocampal and whole-brain atrophy [53]. In addition, amyloid β_{1-42} was found to colocalize with cystatin C in patients with AD. This suggests that a decreased level of cystatin C in the CSF may be associated with increased deposition of amyloid peptides [30, 52]. Moreover, cystatin C CSF levels positively correlate with the levels of tau protein and its phosphorylated form, regardless of the *APOE* genotype, gender, or age of the patient [54]. However, little is currently known about the possible biological interactions between the levels of tau protein and cystatin C. Probably, the expression and secretion of cystatin C increases in response to increased neurodegenerative process [24]. Considering the data on cystatin C binding with β -amyloid, it may also be considered as an attempt to limit amyloid aggregation [55]. CSF cystatin C levels were also compared in patients with AD and vascular dementia, and they were higher in the latter group [56, 57].

So far, studies assessing blood cystatin C levels provide conflicting results. Research by Kálmán *et al.* [56] showed that, despite a higher blood cystatin C level in patients with vascular dementia, it was not significantly different from that in the group of AD patients and the control group. Yaffe *et al.* also analysed the connection between cystatin C and cognitive impairment, indicating that high serum cystatin C levels are associated with cognitive impairment in the elderly [58]. Recently, the study of Cui *et al.* also demonstrated that higher blood levels of cystatin C were positively associated with the cognitive impairment in middle-aged and older Chinese people. Additionally, respondents with higher levels of cystatin C had lower scores on cognitive tests [59]. Consistent with these results, Higgins *et al.* observed that elevated serum cystatin C was associated with 1.2-fold higher prevalence of dementia in a large, diverse sample of middle-aged and older United States adults [60]. Similarly, obese patients undergoing bariatric surgery with elevated cystatin C serum levels obtained lower scores in a neurocognitive assessment prior to the surgery. However, the follow-up improvements in cognitive performance were independent of the observed normalization of cystatin C concentrations [61]. There has also been a report that found plasma cystatin C levels to be lower in AD patients than in controls [46]. However, long-term studies carried out on a large group of patients led to the conclusion that low levels of serum cystatin C precede clinically manifest Alzheimer's disease [62]. A longitudinal analysis by Slinin *et al.* indicates that the relationship between cystatin C and cognitive decline may be

U-shaped, with both very low and very high serum cystatin C concentrations predictive of impaired future cognitive performance [63].

At present, there are no means of identifying the development of the disease from mild cognitive impairment (MCI) to Alzheimer's disease. Cystatin C may prove to be a prognostic factor. Ghidoni *et al.* suggest that a low plasma cystatin C level may be an important indicator of the conversion of mild cognitive impairment to Alzheimer's disease [64]. Therefore, cystatin C was the subject of multiple studies as a potential biomarker or diagnostic tool to identify cognitive impairment and Alzheimer's disease [58, 65, 66]. Reports by Mares *et al.* [67, 68] imply that cystatin C will not be used in differential diagnostics, although it was initially thought that it could play a major clinical role [69].

The relationship of cystatin C and β -amyloid

Cystatin C co-deposits with amyloid- β in AD patients' interstitial, neuropil, and brain blood vessels, as well as in pyramidal neurons located in the III and IV layer of the cerebral cortex [70]. Cystatin C could also be localized in the entorhinal cortex, hippocampus (particularly the CA1 area), the temporal cortex, and, to a lesser extent, in the pyramidal neurons of the frontal, parietal, and occipital lobes, including those in the II layers, which was indicated in immunostaining studies. Moreover, together with cathepsin B, cystatin C was reported to be present in cortical glial cells, endosomes, and lysosomes in the brains of patients with Alzheimer's disease [71]. Cystatin C and amyloid- β co-deposition was also confirmed in the brains of the Rhesus monkey (*Macaca mulatta*) and *Saimiri* (*Saimiri sciureus*) [72]. It has been determined that an overexpression of human cystatin C in the brains of APP transgenic mice reduces β -amyloid deposition and inhibits plaque formation [73]. Both *in vivo* and *in vitro* studies confirmed the binding of cystatin C with soluble amyloid- β . In addition, the inhibition of amyloid- β deposition in transgenic mice with an amyloid precursor protein (APP) mutation were shown [31]. It should be mentioned that the overexpression of human cystatin C does not influence the level of the endogenous murine amyloid- β in the brain [74], or the APP expression. Nevertheless, it might reduce the accumulation of β -amyloid in APP/CysC mice compared to APP mice [31]. Steinhoff *et al.* showed that APP expression in transgenic mice was also associated with an increase in the accumulation of cystatin C in astrocytes, throughout the brain, independently, and before the onset, of amyloid plaque formation [75]. Some findings showed the association of cystatin C and A β demonstrated a specific, saturable, and high-affinity binding

between cystatin C and both A β_{1-40} and A β_{1-42} . Notably, cystatin C association with A β results in a concentration-dependent inhibition of A β fibril formation [76, 77]. It seems that especially the oligomeric form of cystatin C has a potent role in reducing β -amyloid aggregation, possibly due to some similar structural features [55]. Post-mortem studies conducted on patients with Alzheimer's disease and controls revealed that human cystatin C binds to soluble β -amyloid. [78]. Additionally, *in vitro* studies indicate a protective effect of cystatin C on rat hippocampal neurons against the toxic effect of oligomeric or fibrillar forms of β -amyloid [79].

Some evidence demonstrated that cystatin C might participate in the protection processes against neurodegeneration, not only by inhibition of the oligomerization and formation of amyloid- β fibrils, but also by inhibition of cysteine proteases, and induction of autophagy and neurogenesis [25, 28]. *In vitro* studies have shown that cystatin C inhibits B, H, K, L, and S cathepsins [25, 28]. Cathepsins B and D were found in senile plaques of patients with Alzheimer's disease [80]. Cathepsin B has also been found to coexist with cathepsin C in these patients [71]. It has been shown that cathepsin B affects the formation of amyloid- β , possibly by enhancing the activity of β -secretase [81]. Similarly, Hook *et al.* indicated that cathepsin B may act similarly to β -secretase. Therefore, inhibiting its activity reduces the formation of amyloid- β in the course of Alzheimer's disease [82]. The use of cathepsin B inhibitors in an *in vivo* study on transgenic mice with the London APP mutation showed a reduction of memory deficits, amyloid- β in the brain, and a decreased activity of β -secretase. These changes were not observed in the Swedish/London APP mice [83]. Interestingly, increased activity of cathepsin B and D was observed in CysBKO mice. However, decreased cathepsin B activity compared to CysBKO mice occurs in CysBKO/CysC mice (a cross of homozygous CysBKO mice and mice with CysC-overexpression). Thus, the overexpression of cystatin C in CysBKO mice may act as a neuroprotective agent by inhibiting cathepsin activity [84]. Nevertheless, some reports of the anti-amyloid and neuroprotective effect of cathepsin B are in contrast to the above-described reports of its role as a cystatin C inhibitor [85, 86].

The neuroprotective functions of cystatin C

Autophagy is one of the main processes of degradation of long-lived proteins and organelles, and it is essential for the survival of neurons. Autophagic malfunction has been suggested to be an element of the pathogenesis of neurodegenerative disorders, including Alzheimer's disease [87]. Tizon *et al.* showed that cystatin C induces

autophagy and could be considered as a neuroprotective agent [88]. Autophagy activated by cystatin C is manifested as an increase in the degradation of long-lived proteins, which might prove its direct neuroprotection. Moreover, cystatin C showed neuroprotective properties in cell cultures in conditions of cytotoxic changes, where there is nutritional deprivation, colchicine or staurosporine deficiency, or oxidative stress [88]. Under conditions of stress, elevated cystatin C levels induced autophagy by inhibiting the activity of mTOR, which is a negative regulator of autophagy. When autophagy was impeded by 3-methyladenine, an inhibitor, cystatin C no longer had protective functions against the toxicity of nerve cells [88]. It has been demonstrated that the silencing of the gene expression of the Beclin-1 protein, which is an autophagy activator, by siRNA (small interfering RNA) also eliminated the protective effect of cystatin C on serum-deprived cells undergoing apoptosis [88].

Reports from the 1980s and 1990s describe the involvement of cystatin C in the regulation of cell proliferation [89, 90]. Hu *et al.* demonstrated that the secretion of cystatin C mediates APP-induced neural stem/progenitor cell (NSPC) proliferation [91]. Together with fibroblast growth factor 2 (FGF-2), glycosylated cystatin C also affected the stimulation of neurogenesis in the dentate gyrus of the hippocampus of adult rats [92]. The role of cystatin C in neurogenesis has also been confirmed in cystatin C knockout mice, where its basic levels were decreased in the subgranular zone of the dentate gyrus of the hippocampus [93]. In addition, cystatin C can be involved in the process of astrocytic differentiation during brain development in mice [94], and it can affect the differentiation of embryonic stem cells into neural stem cells [95]. The effect of cystatin C on the development of glial cells, including astrocytes, has been confirmed by Hasegawa *et al.* [96].

Interestingly, research shows that various psychiatric drugs may interact with cystatins, leading

to their decreased availability and anti-proteolytic function [34]. While the significance of this phenomenon remains unknown, it could also contribute to the cooccurrence of mental disorders such as schizophrenia, bipolar disorder, or depression with Alzheimer's disease [97].

Taken together, the numerous functions of cystatin C make it a candidate for the development of pharmacological therapeutic interventions for neurodegenerative diseases, such as Alzheimer's disease [98] (Figure 1). However, due to the high costs of the production, the current focus has turned to natural cystatins, such as ovocystatin (Figure 2).

Egg white cystatin – ovocystatin

Egg white cystatin, or ovocystatin, is a type 2 cystatin. It is the most well-known and first isolated protein of the cystatin superfamily [11, 99, 100]. It serves as an inhibitor of the C1 group of cysteine peptidases, including the B, H, K, L, and S cathepsins [33, 99]. Chicken cystatin has very similar biological functions to human cystatin C, which stems from its structure similarities. The amino acid sequence of Ovocystatin is 44% homologous to human cystatin C, whereas there is 62–63% structural homology between the proteins. There is also a strong similarity between the secondary structures of the proteins in solution and in crystalline form [11, 23, 101]. The protein build of ovocystatin comprises a 5-stranded anti-parallel β -sheet wrapping around a central α -helix, and the potential site of β -amyloid binding is the C-terminal hydrophobic sequence formed by amino acids 99 to 115. For cystatin C, the site with the highest β -amyloid binding affinity resides at the analogous structure within the L2 loop and β 5 strand of the protein formed by amino acids 101–117 [102, 103].

The molecular weight of the chicken cystatin is 13.147 kDa, and its single polypeptide chain is comprised of 116 amino acids. There are 2 forms of ovocystatin: a phosphorylated ($pI = 5.6$) and an unphosphorylated form ($pI = 6.5$) [104, 105]. Ovocystatin has a high pH and is thermostable. It loses its inhibitory activity in the process of freezing or lyophilization. This can be deterred in formulations with a concentration of less than 10 μM by adding 20% of glycerol [33, 99, 100]. Chicken egg white cystatin undergoes the 3D swapping phenomenon, which is a mechanism for forming dimers or higher oligomers by exchanging protein domains that remain covalently connected to the core domain through the polypeptide chain [106] and can form aggregates. In this process, it undergoes spontaneous dimerization, followed by the formation of amyloid polymers, which leads to a loss of its biological properties and limits its use

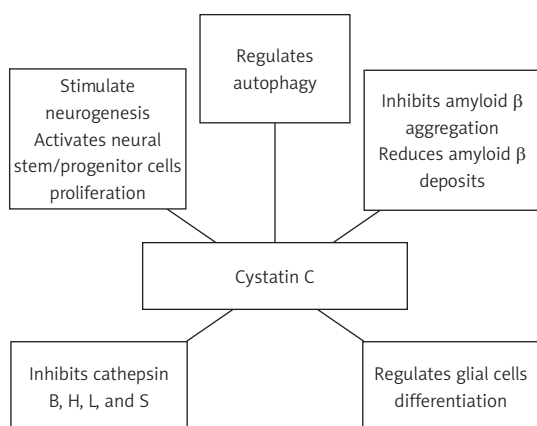


Figure 2. Potential role of cystatin C in neuroprotection

as a potential therapeutic agent. Thus, a safe and effective method of stabilizing ovocystatin while maintaining its inhibitory activity was sought. Enzymatic dephosphorylation of the inhibitor, as well as an addition of trehalose or albumin, proved to help maintain the biological properties and activity of ovocystatin [33, 100].

While the studies on ovocystatin biological properties remain limited, the available data confirm the similarity to the well-evidenced cystatin C in several aspects, with special regard to antimicrobial, anticancer, and neuroprotective properties [107]. The antimicrobial effects of chicken cystatin were studied in relation to various microorganisms. Ovocystatin solution inhibits the growth of various *Escherichia coli* strains *in vitro* [108], while oral intake prevented symptoms of viral gastroenteritis in mouse models [109]. Similarly, ovocystatin combined with interferon- γ (IFN- γ) can be used as a treatment for visceral leishmaniasis in mice, with its mechanism of action related to the increased production of nitric oxide in macrophages [110]. Studies on fungi revealed that ovocystatin inhibits the growth of *Candida albicans* with similar efficacy to fluconazole, whereas no fungal drug resistance to cystatin has been observed [111]. Interestingly, it has been also shown that ovocystatin induces actin cytoskeleton reorganization, as well as apoptosis, in cancer cells [107].

Due to the above-described characteristics of cystatin C and its similarity to ovocystatin, the influence of ovocystatin on cognitive functioning and neurodegeneration has also been explored. Previous research conducted with the use of ovocystatin in animal models revealed that its oral intake correlated with improved cognitive functions assessed with the Morris water maze. Interestingly, these effects were also observed in young rats [112]. Similar results were obtained in the animal model of B6C3-Tg transgenic mice used for the recreation of Alzheimer's disease pathology. Six months of ovocystatin oral intake resulted in significantly improved spatial learning and memory of the experimental group. It has also been revealed that the survival of the mice during the 6-month study period suggests that ovocystatin might be safe to use. Nevertheless, toxicological studies are warranted to confirm this hypothesis [113]. In order to ascertain the underlying molecular mechanisms, a histopathological examination was conducted, revealing that both β -amyloid and tau protein deposits were significantly reduced in the hippocampi of the APP/PS1 mice treated intraperitoneally with ovocystatin [114]. With limited evidence for ovocystatin passage through the blood-brain barrier to the brain compartment, it is also possible that the observed effects were exerted peripherally and indirectly led to the changes in the brain tissue. In another study

it was found that the use of ovocystatin *in vitro* led to reduced aggregation of 1-42 β -amyloid and in turn significantly decreased its toxicity in the PC12 cell line [115].

In conclusion, published studies present data on the endogenous role of cystatin C in the body. Few attempts have been made to administer exogenous cystatin C. Nagai *et al.* applied cystatin C *in vivo* directly into the hippocampus of rats, which led to a destruction of neurons in the dentate gyrus. However, immunohistochemical analysis showed there to be no amyloid- β formation after cystatin C administration [116].

Nutraceutical interventions have gained considerable attention in attenuating cognitive aging and its progression to dementia [117]. Ovocystatin, a cystatin isolated from chicken egg whites, is structurally similar to human cystatin C and its analogous form. Carrying out a series of preclinical studies to assess the effect of an exogenous administration of ovocystatin on cognitive impairment and Alzheimer's disease seems justifiable with the potential for use as a procognitive and routinely used supplement to maintain cognitive function.

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Conflict of interest

The authors declare no conflict of interest.

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An animal model of the procognitive properties of cysteine protease inhibitor and immunomodulatory peptides based on colostrum

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Abstract

Background. The positive effect of human cystatin C on the development of Alzheimer's disease has been reported, as it inhibits the formation of β -amyloid oligomers and amyloidogenesis. Cystatin C has been found to have a neuroprotective effect by inhibiting cysteine proteases, inducing autophagy and neurogenesis. There is a growing interest in the procognitive properties of colostrum-based specimens, which could delay dementia and ameliorate memory deterioration.

Objectives. The aim of the study was to evaluate the influence of ovocystatin and a Coloco peptide complex on the cognitive functions in reference to Colostrinin, using a model of young (4 month-old) and old (10-month-old) Wistar rats.

Material and methods. In the present study, the effects of ovocystatin [100 μ g/rat] and the Coloco peptide [4 μ g/rat] derived from colostrum were assessed with respect to the reference specimen, Colostrinin [4 μ g/rat]. The specimens were administered intraperitoneally and orally for 12 days. Cognitive functions were assessed using the Morris water maze (MWM).

Results. The group of young rats that received ovocystatin orally obtained significantly better results in the MWM compared to the placebo group ($p < 0.05$). Similarly, the group of young rats receiving Coloco orally obtained better results in the MWM compared to the placebo group and to the group of rats receiving Colostrinin ($p < 0.05$). There were no statistically significant differences in the oral and intraperitoneal administration of ovocystatin, Coloco and Colostrinin in the group of old rats.

Conclusions. The obtained results suggest that oral administration of ovocystatin and Coloco has beneficial effects on the cognitive functions of young rats.

Key words: ovocystatin, Coloco, Colostrinin (cln), Morris water maze, cognitive decline

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Population aging results in an increased occurrence of cognitive impairment and dementia. There has been a recent growing interest in biologically active substances present in food such as proteins and peptides. Biologically active substances present in chicken eggs contain cysteine protease inhibitors, including egg white cystatin-ovocystatin.¹ Ovocystatin, with a molecular weight of 13 147 Da, is 44% homologous in terms of the amino acid sequence, 62–63% homologous in terms of the structural sequence and reveals similar biological functions as human cystatin C (Cys C) (molecular weight of 13 343–13 359 Da).^{2–4} As inhibitors of cysteine proteases, cystatins have the ability to inhibit enzymes such as papain or B, H, L and S cathepsins. An imbalance between proteases and their inhibitors may lead to the development of pathological conditions in humans.^{3,5}

Numerous reports on Cys C suggest it has an important role in neurodegenerative diseases, such as Alzheimer's disease. Cys C present in all tissues and body fluids has a wide range of biological properties, such as an antimicrobial and antiviral activity, a role in bone remodeling, neogenesis, modulation of the immune system, and the growth and proliferation of cells.^{6,7} Cys C co-deposits with amyloid- β in the brains of people suffering from Alzheimer's disease and elderly people not diagnosed with dementia. Moreover, when Cys C binds to β -amyloid, it inhibits its oligomerization and amyloidogenesis, and in doing so protects the brain from the toxic effects of β -amyloid.^{6–8} There have also been numerous reports concerning an association between a polymorphism in the Cys C gene (*CST3*) and Alzheimer's disease. As association between the level of Cys C in the blood and cerebrospinal fluid and cognitive impairment in the course of Alzheimer's disease has been found. The serum concentration of Cys C is low when Alzheimer's disease is asymptomatic, and decreases in the cerebrospinal fluid in patients with Alzheimer's disease.^{9,10} Additionally, attention is drawn to other protective mechanisms that Cys C has in neurodegenerative diseases, such as cysteine protease inhibition, inducing autophagy and neurogenesis.^{6,7} Therefore, ovocystatin may have an influence on cognitive functions.

There has also been a growing interest in the proline-rich polypeptide complex (PRP), discovered for the first time in ovine colostrums by Janusz et al.¹¹ It has since been discovered that mammals other than sheep have analogues of colostrum proline-rich polypeptide (PRP) complex as a component of their colostrums.¹² A PRP complex is an important immunomodulator that may induce maturation and differentiation murine thymocytes, promote peripheral blood leukocyte proliferation, induce various cytokines and is a potent antioxidant that significantly reduces 4-hydroxynonenal (4HNE)-mediated cellular damage in cell culture.^{11,13–15} Utilizing high-performance chromatography and mass spectroscopy, several dozen constituent peptides of colostrum PRP later named Colostrinin®, have been identified.¹⁶ Subsequent studies showed

that bovine colostrum may be considered as a suitable source for PRP production by means of methanol/ethanol extraction – Colostrinin or using acetone for fractionation.^{17,18} The later preparation has been named Coloco. The bovine preparations consist predominately of low molecular weight peptides of which both electrophoretic patterns and amino acid composition revealed a high degree of similarity to those of ovine Colostrinin®. A typical for ovine Colostrinin® high level of proline residues (about 20%) and acidic amino acids (about 18%) and low percentages of alanine, glycine, arginine, methionine and histidine, and an absence of tryptophane and cysteine residues was also found to be a characteristic feature for bovine preparations regardless of cow breeds. The employed methods of PRP separation and purification appeared to be reproducible and allow to obtain the PRP preparations very much similar to each other in terms of both chemical composition and biological activity.

The existing in vitro and in vivo studies suggest procognitive properties of Colostrinin. Popik et al. confirmed the positive effect of Colostrinin on cognitive functions in aged rats.^{19,20}

Double-blinded studies carried out on patients with mild and moderate Alzheimer's disease showed that the oral administration of Colostrinin improves or stabilizes the state of the patient.^{21,22} Bilikiewicz et al. (2004) confirmed a positive effect of Colostrinin on cognitive functions as well as everyday functioning.²³ In vitro studies showed that the nonapeptide fragment of the PRP complex (Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro) may directly interact with β -amyloid and hence prevent the formation of toxic deposits.²⁴ Here we present the first study analyzing the Coloco a peptide preparation, separated from cow colostrum according to the procedure described in the patent pending.¹⁸

The aim of the study was to assess the impact of ovocystatin (an analogue of the human cystatin C) and the Coloco peptide complex (obtained from colostrum) with reference to Colostrinin, on the cognitive functions of young and old rats.

Material and methods

Study groups

The study was carried out on male Wistar rats [young (4 month-old) and old (10-month-old)] at the Experimental Laboratory of the Department of Pathology at the Wrocław Medical University. The rats were kept in cages of 2 in standard laboratory conditions, where the room temperature was around 22°C and there was a 12-hour light cycle from 7:00 am to 7:00 pm. The animals were provided with food and water ad libitum.

The rats were assigned to study groups where drugs were administered either intraperitoneally (IP) or orally

(O) at random. The specimens were administered in the following doses per animal: ovocystatin [100 µg], Coloco [4 µg], Colostrinin (CLN) [4 µg] and the placebo [0.9% NaCl] at 0.5 mL/kg over 12 days.

Groups consisted of 7 individuals apart from the subgroups of old rats receiving CLN orally and the placebo both orally and intraperitoneally. In these groups there were 6 rats.

The cognitive functions of the rats were assessed in the Morris water maze. The animals' body mass was assessed prior to and after the cognitive tests. Physical activity was measured before the rats were subjected to the Morris water maze test. All rats were transferred to the study room at least 60 min prior to commencing the test.

Open field test – physical activity

Physical activity was assessed in the open field test (distance, average speed of movement). The rats were placed in an open box individually and were observed for 10 min. The box was cleaned using an alcohol-based agent after each rat.

Morris water maze (MWM)

Each rat was administered the specimen 30 min prior to undergoing the MWM test. The animal was then placed in a round water pool (182 cm wide, 30 cm deep) with a stable platform 10 cm wide, and the rat's head was directed to the wall of the pool. The pool water was at room temperature (22°C) and was colored black with non-toxic paint to obtain a contrast between it and the white fur of the rat. Three days of adaptation and 9 days of acquisition training preceded the probe trial. The animals began each session during acquisition training from a different position (N, S, W, E). If the rat reached the platform before 120 s, it remained on the platform for 5 s and then was removed from the pool. On the other hand, if the rat did not reach the platform, it was guided to it, and remained on it for 20 s before being taken out of the pool.

On day 13 (probe trial) the test was carried out without the platform. On day 14, (probe with a visible platform) the behavior of the rat when the position of the platform was changed (visible 1 cm above the water level) was tested. The measurement system that was applied included the Sony Color SSC-DC378P camera and a workstation, equipped with v. 2.5 of SMART software (PanLab, Spain).

Reagents

A proline-rich polypeptide complexes (PRP) Colostrinin and Coloco were isolated from bovine colostrum according to Kruzel et al. and Polanowski et al., respectively.^{17,18}

Ovocystatin, monomeric form, was separated from chicken egg white as described in Gołąb et al.²⁵

Statistical analysis

Data for the group of young and old rats was analyzed separately. In addition, control study subgroups were compared with each other. Ovocystatin was analyzed independently of Coloco and CLN. The oral and intraperitoneal routes of administration were also analyzed independently of one another. Parameters that changed with time (body weight, physical activity, Morris water maze parameters) were statistically analyzed using repeated measures ANOVA. An analysis of multiple repetitions was used as a post-hoc test with the Holm correction. On the day of running the Morris water maze test, the parameters were analyzed using classic ANOVA (1 or 2-way ANOVA). In two groups, depending on the results of the F test for the equality of variances, statistical analysis was carried out using the Student's t-test or the Welch test. The level of statistical significance was set at $\alpha = 0.05$. Statistical analysis was carried out using v. 3.0.2 of the R statistical software for Windows (The R Foundation for Statistical Computing, Vienna, Austria), and v. 12.7.7 of MedCalc for Windows (MedCalc Software, Mariakerke, Belgium).

The study was carried out with the permission of the I Local Ethics Committee for Animal Experiments in Wrocław at the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences in Wrocław (permission no. 46/2012;74/2012;75/2012).

Results

The effect of using the formulations on body mass

The administration of ovocystatin intraperitoneally to young and old rats had no effect on their weight in the different subgroups compared to the placebo ($p > 0.05$). However, the body mass of young rats increased with time ($p = 0.001$), whereas the body mass of old rats decreased with time when ovocystatin was administered intraperitoneally ($p = 0.007$). Administering ovocystatin orally to young and old rats did not affect their body mass. However, the body mass of animals in both age groups increased with time during the study period ($p = 0.002$).

An intraperitoneal administration of Coloco and CLN did not affect weight in the subgroups of young and old rats. A significant association between body mass and group is associated with the loss of body mass of the old rats receiving the placebo ($p = 0.039$). Administering the formulations orally had no effect on the body mass of young and old rats either. There was a significant interaction between time and the formulation used in the group of old rats receiving CLN, which was caused by a difference in body mass measurements in the first and second recordings (data not shown).

Assessing locomotor activity in the open field test

The group of young rats assigned to the subgroup receiving ovocystatin intraperitoneally was significantly slower ($p = 0.006$) and covered a significantly shorter distance ($p = 0.006$) than animals in the placebo group. In the group of old rats, no differences in the average speed and distance travelled were noted.

The subgroup of young rats receiving ovocystatin orally was significantly slower ($p = 0.0017$) and traveled a significantly shorter distance ($p = 0.0017$) than animals in the placebo group, and old rats from the subgroup receiving ovocystatin orally were significantly faster ($p = 0.032$) and moved a longer distance ($p = 0.03$) than the placebo group.

The groups of young and old rats receiving Coloco and CLN orally did not differ in terms of their average speed and distance traveled ($p > 0.05$). The group of young rats receiving Coloco in the form of an intraperitoneal injection was significantly slower and travelled a shorter distance than the group receiving CLN ($p = 0.0017$) or the placebo ($p = 0.0017$). The group of old rats receiving

Coloco intraperitoneally had significantly higher average speed values than the CLN group ($p = 0.027$) and lower than the placebo group ($p = 0.046$). The group of old rats from the placebo subgroup travelled a longer distance than those from the CLN ($p = 0.027$) and Coloco ($p = 0.045$) subgroups, where all the animals received the formulations intraperitoneally (Fig. 1–2).

The effect of the formulations on cognitive function in the Morris water maze

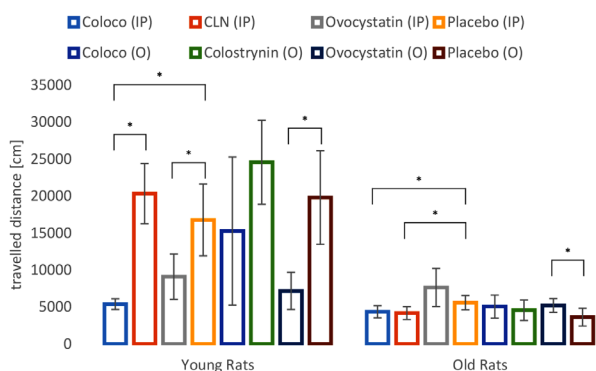
Acquisition training

In the group of young rats, intraperitoneal and oral administration of ovocystatin, Coloco and CLN did not shorten the time of the animals to find the platform, in comparison to the placebo group ($p > 0.05$). It was noticeable that the length of time it took the rats, both young and old, to find the platform decreased with each day of training ($p < 0.05$). The group of young rats receiving Coloco intraperitoneally was significantly slower than the group receiving CLN ($p = 0.022$). However, these subgroups were not differ significantly from the control group. There were no differences between the remaining subgroups of young and old rats ($p > 0.05$). Wishaw's error, which defines with what precision a given individual finds the hidden platform, did not show any significant differences in the subgroups of young and old rats receiving oral and intraperitoneal specimens. The parameter showed an upward trend over training time in all groups receiving specimens intraperitoneally and in the subgroup of young rats receiving ovocystatin intraperitoneally (data not shown).

Probe trial

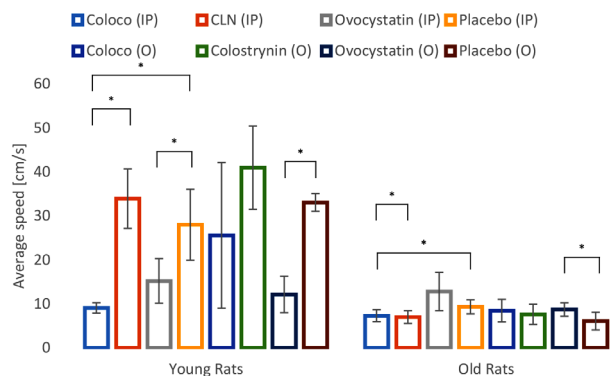
There was a significant difference in the percentage of time spent in the target sector (PT%) and the percentage of the distance covered in the target sector (D%) between the group of young rats receiving ovocystatin orally and the

Fig. 1. Open Field Test – travelled distance



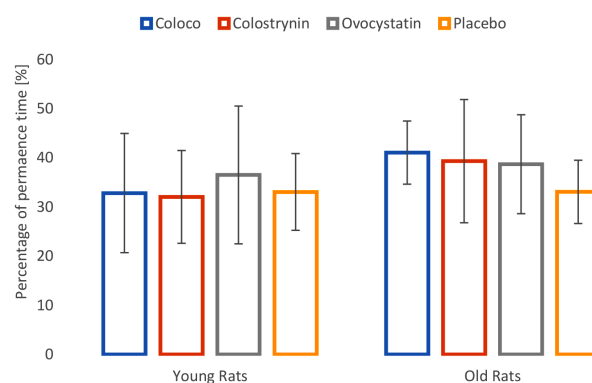
* $p < 0.05$; IP – intraperitoneal administration; O – oral administration. Data are expressed as mean \pm SEM.

Fig. 2. Open Field Test – average speed

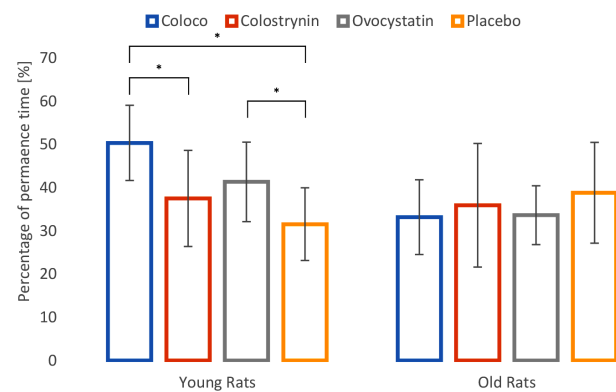


* $p < 0.05$; IP – intraperitoneally administration; O – orally administration. Data are expressed as mean \pm SEM.

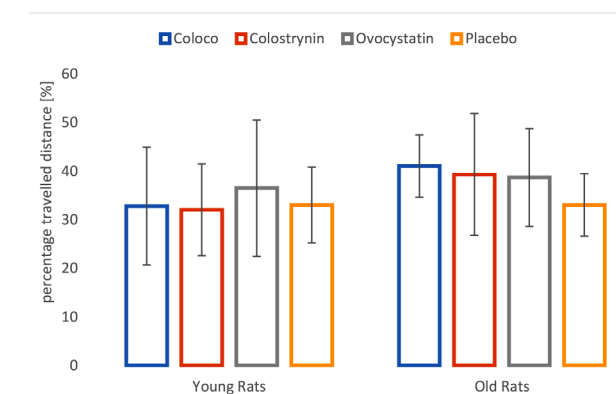
Fig. 3. Morris Water Maze – probe trial. Percentage of permanence time at target sector (PT%) – IP administration



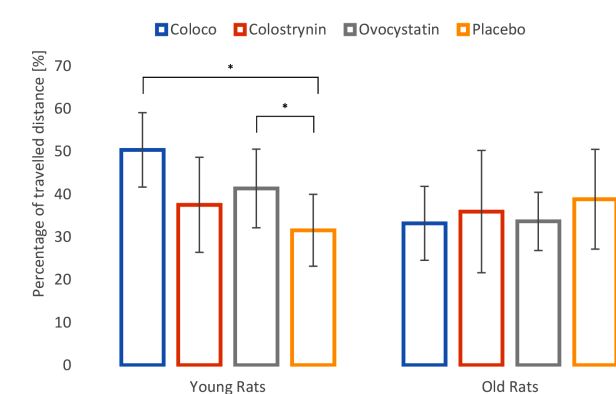
Data are expressed as mean \pm SEM.

Fig. 4. Morris Water Maze – Probe trial. Percentage of permanence time at target sector (PT%) – Orally administration

* $p < 0.05$; Data are expressed as mean \pm SEM.

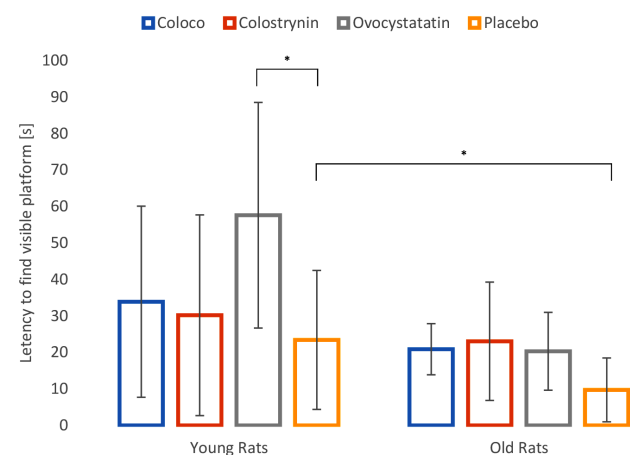
Fig. 5. Morris Water Maze – Probe trial. Percentage of permanence travelled distance at target sector (D%) – IP administration

Data are expressed as mean \pm SEM.

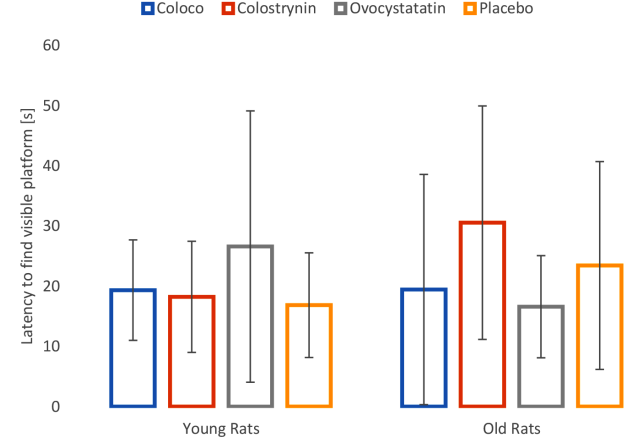
Fig. 6. Morris Water Maze – Probe trial. Percentage of permanence travelled distance at target sector (D%) – Orally administration

* $p < 0.05$; Data are expressed as mean \pm SEM.

placebo group ($p = 0.0027$ and $p = 0.010$ respectively). The group of young rats receiving Coloco orally differed significantly from both the placebo group ($p = 0.0048$) and the group receiving CLN ($p = 0.0415$) in terms of the percentage of time spent in the target sector (PT%). A significant difference in the percentage of distance covered in the tar-

Fig. 7. Morris Water Maze - Probe with visible platform. Latency to find visible platform - IP administration

* $p < 0.05$; Data are expressed as mean \pm SEM.

Fig. 8. Morris Water Maze - Probe with visible platform. Latency to find visible platform - Orally administration

Data are expressed as mean \pm SEM.

get sector (D%) in the subgroup of young rats receiving Coloco orally compared to the placebo ($p = 0.0037$) was noted (Fig. 3–6).

Probe with visible platform

The subgroup of young rats receiving ovocystatin intraperitoneally took longer to find the platform when compared to the placebo group ($p = 0.042$). In the case of the old rats, there were no differences between the subgroups ($p > 0.05$) regarding the time needed to find the platform. Old rats found the platform faster than young rats in the subgroups of rats receiving the placebo ($p = 0.025$). No differences were found between the groups of young and old rats receiving ovocystatin orally ($p > 0.05$) (Fig. 7–8).

No differences were found in the groups of young and old rats receiving Coloco, CLN and the placebo ($p > 0.05$). The subgroup of old rats receiving the placebo intraperitoneally found the platform faster than young rats

($p = 0.032$). The oral administration of Coloco, CLN and the placebo did not lead to any significant changes in the measured parameters ($p > 0.05$) (Fig. 7–8).

Discussion

The present results suggest ovocystatin obtained from chicken eggs and colostrum-based Coloco may have potential procognitive properties when given orally to the group of young rats. Unexpectedly, the administration of ovocystatin at a dose of 100 µg/rat and Coloco at 4 µg/rat resulted in an increased ability to concentrate in young individuals, even though it was speculated before carrying out the study that these specimens would have a positive effect on the cognitive functions of older individuals. The MWM and a modified scheme of this test, used in previous studies concerning CLN (a reference specimen in this study), were used to assess the procognitive properties of the studied specimens.¹⁹ The MWM allows the assessment of hippocampal-dependent spatial memory. The applied model of dementia allows the assessment of the activity of the studied specimens, and their influence on the deterioration, both natural and pathological, of an organism's cognitive functions.^{19,20} It also allows the identification of the influence of the specimens on the ability to concentrate in the case of young individuals. Although the results obtained in an animal model may not have direct implications in humans, it has been shown that the use of CLN in patients leads to cognitive improvements.^{21–23}

It is also important to assess locomotor activity and exclude possible effects of the aging process on it, using the open field test. This test is also used in studies concerning anxiety. In the group of old rats, as opposed to the group of young rats, no differences were found, suggesting that the aging process did not affect locomotor activity. The observation of a certain locomotor activity in the group of young and old rats shows whether the animals have a sufficient physical condition to undergo further studies in the MWM. This study did not evaluate the effect of a given specimen supplementation on locomotor activity. However, in this study, the lack of a difference in the average swimming speed during the MWM workout between groups receiving the specimens and the placebo is consistent with earlier studies using CLN, and suggests a lack of effect of specimen supplementation on physical activity.¹³

Due to a significant structural similarity of ovocystatin – a chicken cystatin and human Cys C, an attempt was made to determine the optimal route of administration of ovocystatin and its impact on the cognitive function.²⁶ To date, the effect of direct administration of cystatin into the hippocampus was analyzed by Nagai et al. The study revealed a loss of neurons in the dentate gyrus in the brains of Sprague-Dawley rats, although an immu-

nohistochemical analysis showed no β -amyloid formation.²⁷ However, in vivo studies in the case of an overexpression of human Cys C in the brains of transgenic mice with a mutation in the amyloid precursor protein have shown that Cys C inhibits the deposition of β -amyloid. It may also regulate processes of β -amyloidosis in the brain, and may therefore have potential therapeutic use.^{28,29} In the present study, ovocystatin administered intraperitoneally was not found to have procognitive properties.

The results of the MWM carried out without a platform may be used as the basis for further studies regarding the procognitive effect of ovocystatin administered orally.

So far, the correlation of Cys C with cognitive functions has been examined using the MWM, where the relationship of Cys C with cathepsin B with regard to the level of β -amyloid was studied in a mouse model of Alzheimer's disease.³⁰ It is one of few studies analyzing Cys C and indicates its negative impact on the disease, which is contrary to the results of the present study.

In addition, the results obtained on the day when the platform was visible showed that young rats receiving ovocystatin intraperitoneally looked for the platform longer, especially when it was positioned in the opposite zone. This may mean that the animals remembered the previous location of the platform and spent more time trying to find it. A similar tendency, although not statistically significant, was seen in the group of young rats receiving ovocystatin orally.

World reports on the effects of the proline rich polypeptide complexes on cognitive functions indicate their procognitive properties both in humans and animals.^{19–23} The obtained results of the MWM without the platform suggest its effectiveness when given orally. Furthermore, no procognitive function was found in the group of old rats. The results of the group of young rats suggest that it may affect concentration.

The study has a number of limitations. One of these is a small study group. Also, the study was carried out using only 1 dose of each of the specimens. Possibly, increasing the range of dosages as well as the duration of use will contribute to achieving more promising results. Additionally, the study is limited to an animal model, which is subject to physiological aging and dementia. Therefore, it seems reasonable to continue the study using an animal model of Alzheimer's disease. Further studies concerning ovocystatin and Coloco in terms of cognitive disorders and neurodegenerative diseases are required. Taking into account a positive trend found in this study in the group of old rats (although not statistically significant), it seems necessary to continue the study using this age group.







In conclusion, the obtained results indicate procognitive properties of ovocystatin and Coloco and may contribute to further research concerning the impact of these specimens on cognitive functions and their mechanisms of action.

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Article

Ovocystatin Induced Changes in Expression of Alzheimer's Disease Relevant Proteins in APP/PS1 Transgenic Mice

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Abstract: Background: Ovocystatin is marked by structural and biological similarities to human cystatin C, which plays an important role in the course of neurodegenerative diseases. Recently, it has been shown that ovocystatin might prevent aging-related cognitive impairment in rats and reduce memory decline in an APP/PS1 mice model. Thus, this study aimed to assess the effect of ovocystatin on histopathological changes in APP/PS1 mice. Materials and methods: Ovocystatin was administered intraperitoneally for four weeks (40 µg/mouse) to 35-weeks-old transgenic (AD, n = 14) and wild type (NCAR, n = 15) mice (stock B6C3-Tg(APP^{swe}, PSEN1^{dE9})85Dbo/Mmjax). A histopathological evaluation comprised antibodies directed against β-amyloid (1:400, SIG-39320-1000, Covance) and Tau (1:4000, AHB0042, Invitrogen). Three regions of the hippocampus—the dentate gyrus (DG) and the cornu ammonis (CA1 and CA3)—were analyzed by immunohistochemistry in each animal. All differences are expressed as percentage relative to the control group. Results: The main results showed that the percentage of immunoreactive area of β-amyloid, tau protein deposits in APP/PS1+ovCYS was decreased in DG, CA1, and CA3 regions compared with the APP/PS1 control, respectively (*p* < 0.05). Conclusions: Ovocystatin caused significant changes in the expression pattern of all investigated proteins in hippocampal tissues both in APP/PS1 and NCAR mice.

Keywords: ovocystatin; chicken cystatin; cystatin C; Alzheimer's disease; mice

1. Introduction

Alzheimer's disease (AD), as a progressive and neurodegenerative disorder, has substantial consequences for a patient's quality of life and their carers. Heretofore, the effective treatment is still unknown and current treatments have been restricted only to

cholinesterase inhibitors (rivastigmine, donepezil) and the antagonist of N-methyl-D aspartate (NMDA) receptor (memantine), which only affect the symptoms [1–3]. Moreover, the etiology of AD is associated with many factors, such as genetic, biological, or social ones [4]. It has been also suggested that inflammation plays an important role in the course of AD and bacterial, viral, or fungal infections might be crucial in the pathogenesis [5]. Indeed, recently published data [6] revealed that oral *Porphyromonas gingivalis* infection resulted in mice brain colonization and increased production of β -amyloid fragments 1–42 ($A\beta_{1-42}$). Moreover, gingipains—the toxic proteases, are identified in AD patients' brains and linked with protein tau and ubiquitin-related pathology. Nevertheless, β -amyloid ($A\beta$), apolipoprotein E (APOE), and protein tau are still considered the substantial elements which contribute to Alzheimer's disease pathophysiology [3]. Several lines of evidence support that mutations in amyloid precursor protein (APP) and presenilin 1 (PSEN1) and 2 (PSEN2) genes lead to the development of the disease and production of toxic $A\beta$, especially in patients with early-onset autosomal dominant familial Alzheimer's disease (FAD) [7,8]. From the neuropathological point of view, AD is characterized by amyloid plaques and neurofibrillary tangles (NFTs), followed by neurodegeneration with synaptic and neuronal loss causing macroscopic atrophy [9]. AD can be divided into three stages, according to the degree of cognitive impairment [10]. Memory loss, cognitive impairments, and behavioural changes are the main clinical manifestations of AD, affecting the daily activities of the affected individuals [11]. It has been evaluated that AD will affect 131 million people worldwide by 2050 and will cause over US \$2 trillion in economic losses by 2030 [12]. Thus, novel therapeutic approaches concerning anti-amyloid therapy, anti-tau therapy, anti-neuroinflammatory therapy, neuroprotective agents including N-methyl-D-aspartate (NMDA) receptor modulators, and brain stimulation are still being searched [13]. Moreover, different tested bioactive compounds also have the potential for clinical application. Some proteins, carbohydrates, vitamins, fats, and oils may synadditively confer overwhelming protection against neurodegenerative diseases by modulating the activities of these critical enzymes of physiological importance [14].

Multiple lines of research have revealed that cysteine proteases play an important role in AD pathology [15]. It has been widely demonstrated that cysteine protease inhibitor-cystatin C (Cys C) might be a potential target for Alzheimer's disease treatment [16]. Cys C has been found in all mammalian body fluids, such as cerebrospinal fluid (CSF), blood plasma, and all mononuclear cells. In the brain it is expressed by neurons, astrocytes, endothelial, and microglial cells (for review see: [17]). Cys C has a broad spectrum of biological functions ranging from modulation of inflammatory response, antibacterial and antiviral properties, to inhibition of tumor metastasis [18–20]. Interestingly, numerous studies have demonstrated that Cys C plays a crucial biological role in neurodegenerative disorders, especially in the pathophysiology of Alzheimer's disease [21]. It has been shown that Cys C co-deposits with $A\beta$ in Alzheimer's disease patients' brains [22] and the association between cystatin C and $A\beta$ demonstrate a specific, saturable, and high-affinity binding between cystatin C and both $A\beta_{1-42}$ and $A\beta_{1-40}$ [23]. Additionally, in vitro studies reported inhibitory properties of Cys C against fibril formation and oligomerization [24]. Studies by Kaur and Levy [20] and Gauthier et al. [21] elaborate on the neuroprotective roles of Cys C, including the inhibition of cysteine proteases, such as cathepsins B, H, K, L, and S, the induction of autophagy, and the regulation of cell proliferation which is linked to the induction of neurogenesis. Similarly, oral administration of the cysteine protease inhibitor E64d improved memory deficit. Furthermore, reduced brain $A\beta_{40}$ and $A\beta_{42}$, amyloid plaque, and brain cathepsin B activity were observed [25].

In the light of these findings, a need of developing new therapeutic strategies comprising the prevention and treatment of Alzheimer's disease has been highlighted. Thus, our study focused on ovocystatin (ovCYS), which is the best-characterized type 2 cystatin protein. Ovocystatin has been used in a series of experimental studies as a model protein representing the cystatin superfamily [26]. Likewise, cystatin C inhibits a broad range of lysosomal cathepsins, including cathepsin B, H, K, L, and S [27]. The protein is highly

homologous to its human counterpart cystatin C, (62% structural similarity) and has similar biological properties [28]. In addition, it has been revealed that ovocystatin is characterized by relatively low immunogenicity [29]. Moreover, in contrast to cystatin C it can be easily obtained from chicken eggs on a large scale [30].

Recently, Stańczykiewicz and colleagues have shown that ovocystatin has beneficial properties for cognitive functions in young rats [31], and might prevent aging-related cognitive impairment in older animals [32] as well as reduce memory decline in the APP/PS1 mice model [33]. Moreover, it has been shown that six-months of ovocystatin administration in drinking water may become a safe, effective, and well-tolerated approach in the prevention of cognitive decline in APP/PS1 mice [33]. For these reasons, the protein seems to be a suitable tool for studying the role of cystatins in the pathophysiology of neurological disorders. Indeed, the prevention of neurodegeneration is an important aspect of modern medicine. Addressing several limitations in previous studies, related to a lack of morphological, biochemical, and immunohistochemical studies, our research can provide the first data of more reliable insights into the biological properties of ovocystatin. Heretofore, it has been shown that ovocystatin may inhibit the deterioration of cognitive functions without indicating crucial properties of ovocystatin, related to neurodegenerative processes. Hence, this study aimed to assess the effect of ovocystatin on histopathological changes in APP/PS1 mice.

2. Materials and Methods

2.1. Reagents—Isolation and Characterization of Ovocystatin

Egg white homogenate was applied on an affinity chromatography column containing S-carboxymethylated papain-Sepharose 4B. Ovocystatin was eluted with 50 mM K_3PO_4 containing 0.5 M NaCl at pH 11, dialysed against 50 mM NH_4HCO_3 and lyophilized. The anti-papain activity of the inhibitor was measured calorimetrically against α -N-Benzoyl-DL-arginine β -naphthylamide (BANA) as a substrate [34]. The purity of ovocystatin was checked by SDS-PAGE in 12% gel under reducing conditions [35]. Based on SDS-PAGE electrophoresis, the inhibitor was pure and not aggregated. A more detailed description of ovocystatin preparation was presented in another paper [33].

2.2. Animals—APP/PS1 Mice

Male APP/PS1 transgenic mice used for immunohistochemical analysis were purchased from Jackson Laboratory, Bar Harbor, ME, USA. These mice display the development of A β deposits by six months of age and express the mouse/human APP_{swe} (K595N/M596L) and exon-9 deleted presenilin 1 (deltaE9) [36,37]. The mice were housed (three to four per cage) under standard conditions (12:12 light-dark cycle with lights on at 7:00 a.m.; temperature 21 ± 2 °C) and with free access to pelleted mouse chow and drinking water.

2.3. Animals Grouping, Intervention, and Sample Preparation

Thirty-five-week old APP/PS1 (B6C3-TG (APP_{swe}, PSEN Δ E9)85Dbo/J, initial body weight approximately 27 g; n = 14) and age-matched non-carrier (NCAR) control (initial body weight approximately 28.66 g; n = 15) were randomly assigned into control and treatment (ovCYS) groups, so at the end, there were four experimental groups: APP/PS1 (vehiculum, n = 7), APP/PS1 + ovCYS (n = 7), NCAR (vehiculum, n = 7), NCAR + ovCYS (n = 8). Mice were injected intraperitoneally for four weeks (five days every week with a two day gap). Ovocystatin was given at the dosage of 40 μ g/mouse, corresponding to 100 μ L of working solution, while control groups received vehicle injections (0.9% saline) starting at the age of 35-weeks. After four weeks of administration, mice were deeply anesthetized with a cocktail of ketamine and xylazine (50–100 and 5–10 mg/kg i.p. respectively) and perfused transcardially with 0.9% saline solution. Brains were dissected and cut into left and right hemispheres. The right hemisphere was fixed in 4% phosphate-buffered formalin and embedded in paraffin afterward.

All animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Local Ethical Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.4. Immunohistochemistry

Immunohistochemical reactions were performed on 4- μ m-thick paraffin sections using Autostainer Link48 (Dako, Glostrup, Denmark). Tissue sections were deparaffined, rehydrated and antigen retrieval was carried out by treating the slides with EnVision FLEX Target Retrieval Solution (97 °C, 20 min; pH 9) using a PT-Link. The activity of endogenous peroxidase was blocked by 5 min. incubation with EnVision FLEX Peroxidase-Blocking Reagent (Dako). Afterward, primary antibodies (diluted in EnVision FLEX Antibody Diluent (Dako)), β -amyloid (mouse Mo, anti-human; 1:400, SIG-39320-1000, Covance, Princeton, NJ, USA), and tau (mouse Mo, anti-human, mouse; 1:4000, AHB0042, Invitrogen, Waltham, MA, USA) were applied for 20 min. Next, slides were incubated with EnVision FLEX/HRP (20 min). 3,3'-diaminobenzidine (DAB, Dako) was utilized as the peroxidase substrate and the sections were incubated for 10 min. Finally, all sections were counterstained with EnVision FLEX Hematoxylin (Dako) for 5 min. After dehydration in graded ethanol concentrations (70%, 96%, 99,8%) and xylene, slides were closed with coverslips in Dako Mounting Medium (Dako).

2.5. Image Analysis

The sections were evaluated under the BX-41 light microscope Olympus, Tokyo, Japan and were collected serially. For image analysis of immunohistochemistry, 3 coronal sections taken from the middle (bregma; -1.82 mm) hippocampus of each mouse was analyzed. All immunoreactive areas of plaque were quantitatively analyzed using ImageJ, version 1.49 (NIH, Bethesda, MD, USA). Quantification was performed under $40\times$ magnification. Image processing included background correction, color deconvolution, and adjustment to obtain the true positive DAB signal. The average pixel density within the specific region of the hippocampus was then recorded for further calculations. The two different experimenters were blinded to genotype and treatment during image collecting and processing. Data are expressed as the percentage of the hippocampal region occupied by the positive DAB signal.

2.6. Statistical Analysis

Data were expressed as mean \pm standard deviation and analyzed using IBM SPSS Statistics ver. 23 (IBM Corporation, Armonk, NY, USA). Two-way ANOVA with Bonferroni post-hoc comparisons was used to determine the differences between groups. Genotype/treatment and ROI were the independent variables and the percentage of the area with positive immunoreactivity was the dependent variable in two-way ANOVA. Statistical significance was set at $p < 0.05$.

3. Results

The effects of ovocystatin administration on amyloid plaque and pathological tau protein deposits were studied by immunohistochemical analysis of hippocampal slices in a mouse model of Alzheimer's disease. The burden of β -amyloid and tau protein deposition in the hippocampus of transgenic (APP/PS1) and non-carrier (NCAR) mice treated with ovocystatin was analyzed. A quantitative analysis was repeated twice for precision. The average value of two measures was obtained in each animal. Three regions of the hippocampus (region of interest, ROI)—DG, CA1, and CA3—were analyzed in each animal. All differences are expressed as a percentage relative to the control group (Figures 1 and 2).

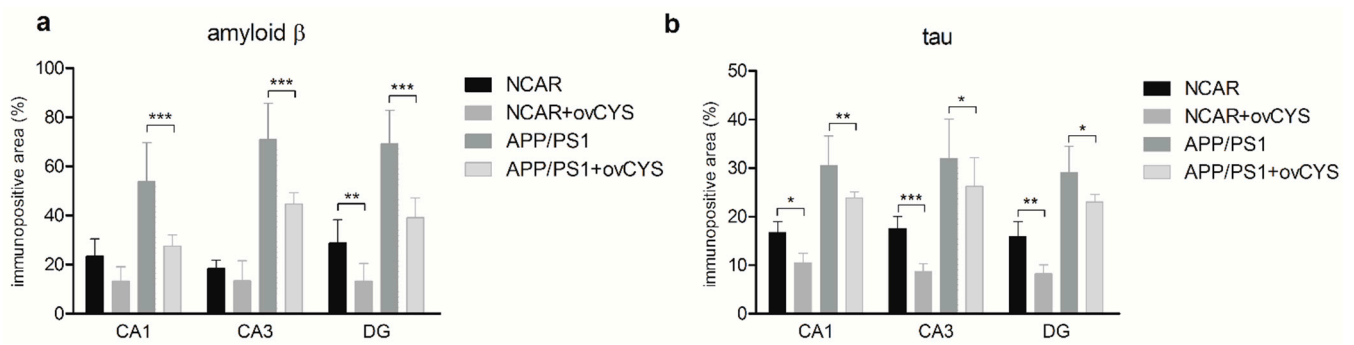


Figure 1. Immunoreactive area (percentage of the total area measured) of β -amyloid (a) and tau (b) burden in three hippocampal regions. (a) the burden of β -amyloid significantly decreased in the ovocystatin-treated transgenic group within all analyzed regions of the hippocampus, (b) deposits of misfolded tau protein significantly decreased in both non-carrier and transgenic ovocystatin-treated groups within all analysed hippocampal regions. NCAR—non-carrier group, NCAR+ovCYS—ovocystatin-treated non-carrier group, APP/PS1—transgenic group, APP/PS1+ovCYS—ovocystatin-treated transgenic group. Statistical significance of treated ovCYS versus untreated groups was indicated as * at $p < 0.05$, ** at $p < 0.01$, and *** at $p < 0.001$ within the respective hippocampal region.

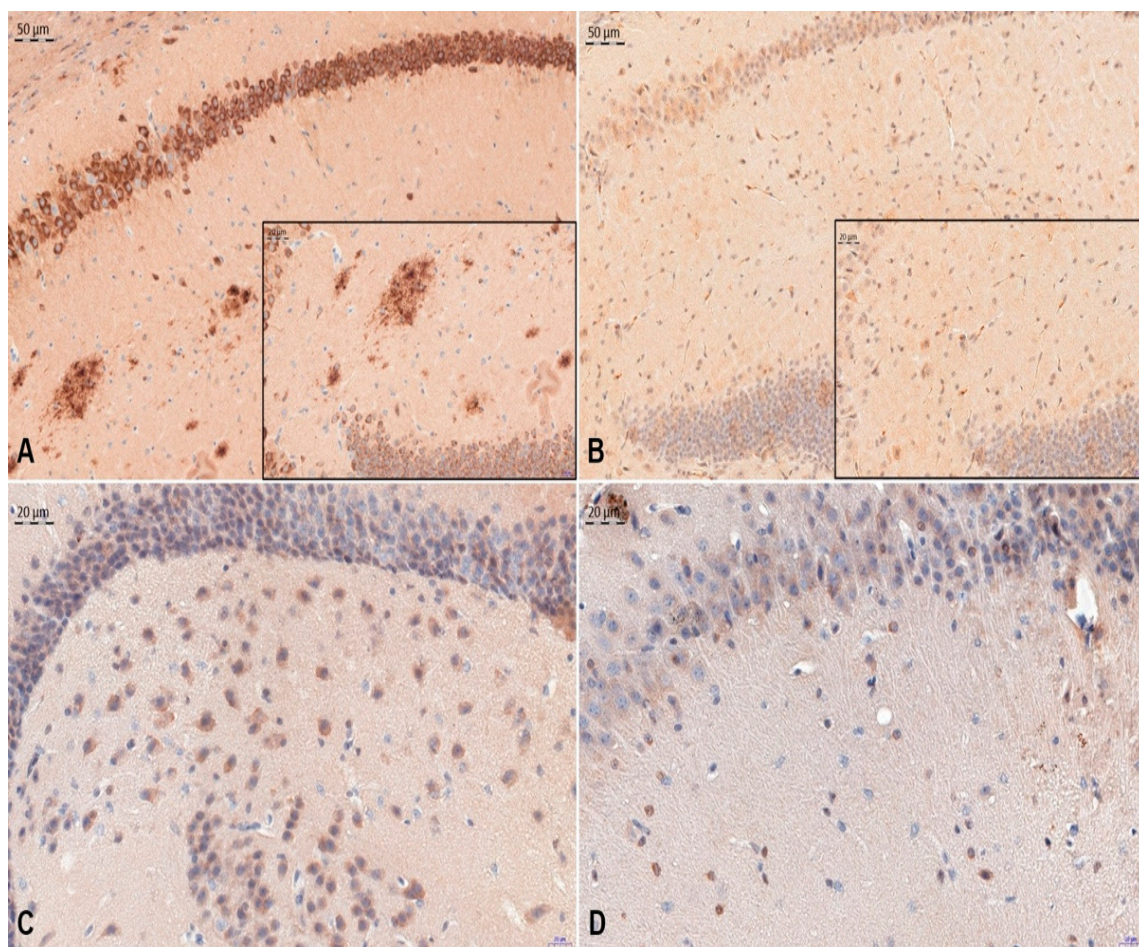


Figure 2. Representative images of immunohistochemical reactions indicating β -amyloid (A,B) and TAU-5 (C,D) antigen expression were carried out on APP/PS1 + ovCYS group (A,C) and NCAR control (B,D) mouse brain. Nuclei are stained using hematoxylin. Magnification $\times 200$ (A,B) and $\times 400$ (C,D and insert).

The percentage of the positive immunoreactive area of β -amyloid (Figure 1a) in ovocystatin treated transgenic mice (APP/PS1 + ovCYS, $n = 7$) was decreased by 30.14 in DG (39.14 ± 3.00 vs. 69.29 ± 5.15 , $p < 0.001$), 26.41 in CA1 (27.57 ± 1.69 vs. 53.98 ± 5.94 , $p < 0.001$) and 26.14 in CA3 (44.71 ± 1.74 vs. 70.86 ± 5.59 , $p < 0.001$), compared with the APP/PS1 control ($n = 7$), respectively. The percentage of the positive immunoreactive area of β -amyloid in ovocystatin treated non-carrier mice (NCAR + ovCYS, $n = 8$) was decreased by 15.51 in the dentate gyrus (13.25 ± 2.55 vs. 28.76 ± 3.61 , $p < 0.01$), 10.14 in CA1 (13.25 ± 2.07 vs. 23.39 ± 2.67 , $p > 0.05$) and 5.00 in CA3 (13.38 ± 2.88 vs. 18.38 ± 1.29 , $p > 0.05$), compared with the NCAR control ($n = 7$), respectively. The strong statistically significant effect of treatment ($F_{(3,75)} = 125.0$, $p < 0.0001$) and ROI ($F_{(2,75)} = 6.573$, $p = 0.0023$), as well as the strong significant interaction between treatment and ROI ($F_{(6,75)} = 3.059$, $p = 0.0099$), was observed in the analysis. The interaction resulted from the difference (decrease) of the positive signal observed in the dentate gyrus, which was bigger in the ovCYS treatment groups.

The percentage of the positive immunoreactive area of tau protein deposits (Figure 1b) in the APP/PS1 + ovCYS group ($n = 7$) was decreased by 6.14 in the dentate gyrus (23 ± 0.62 vs. 29.14 ± 2.00 , $p < 0.05$), 6.71 in CA1 (23.86 ± 0.46 vs. 30.57 ± 2.28 , $p < 0.01$) and 5.71 in CA3 (26.29 ± 2.22 vs. 32 ± 3.06 , $p < 0.05$), compared with the APP/PS1 control ($n = 7$), respectively. The percentage of the positive immunoreactive area of tau protein deposits in the NCAR + ovCYS group ($n = 8$) was decreased by 7.61 in the dentate gyrus (8.25 ± 0.65 vs. 15.86 ± 1.18 , $p < 0.01$), 6.21 in CA1 (10.5 ± 0.68 vs. 16.71 ± 0.87 , $p < 0.05$) and 8.82 in CA3 (8.75 ± 0.56 vs. 17.57 ± 0.92 , $p < 0.001$), compared with the NCAR control ($n = 7$), respectively. The statistically significant effect of treatment ($F_{(3,75)} = 120.3$, $p < 0.0001$) was observed in the analysis.

4. Discussion

To our knowledge, this is the first study evaluating the histopathological changes in brain tissue of APP/PS1 mice after the intraperitoneal administration of ovocystatin. To date, only several studies have aimed to address alternations of cognitive functions after ovocystatin supplementation. Indeed, recently published data by Stańczykiewicz and colleagues [33] revealed that ovocystatin administered for six months in drinking water at a dose of 40 $\mu\text{g}/\text{mouse}$ reduces memory deficits in APP/PS1 transgenic mice. Moreover, it was noted that ovocystatin was given orally and intraperitoneally to improve cognitive functions in young rats [31]. The potential protective effect of ovocystatin administered intraperitoneally on age-related cognitive impairments in rats was also determined by Stańczykiewicz et al. [32]. Nevertheless, the obtained results were not statistically significant, but highlight the potential role of ovocystatin in neurodegenerative disorders. Additionally, it has to be mentioned that prolonged ovocystatin administration did not affect physical activity and might be a safe and effective intervention for Alzheimer's disease. However, the exact mechanisms of action by this mode of ovocystatin administration remain unclear.

We do not know whether or not intact ovocystatin had crossed the blood-brain barrier (BBB) and eventually reached the neuronal tissues. Perhaps ovocystatin might have reached the neuronal compartment by some mechanisms that are not well defined yet. The passage of proteins within extracellular vesicles from circulation to the brain and the other way out is well established and is implicated in the pathogenesis of neurodegenerative disorders [38–40]. It is also plausible that the protein did not reach the brain tissues but acted by peripheral interactions with the immune system. There is compelling evidence that apart from CNS inflammation, the peripheral immune response may be involved in the progression of neurodegenerative diseases [41].

Thus, for the first time, we performed the histopathological evaluation taking into account three regions of the hippocampus—the dentate gyrus (DG), CA1, and CA3. It comprised antibodies directed against β -amyloid and Tau, which reflects an apparent neuropathological change in the hippocampus—the intraneuronal accumulation of A β 42

and microtubule stability, respectively. For example, in Alzheimer's disease, tau accumulates in the somatodendritic compartment [42,43]. Interestingly, we found that ovocystatin ameliorates hippocampal neurodegenerative changes, and thus might be beneficial for Alzheimer's disease treatment. Indeed, our findings showed that the percentage of positive immunoreactive areas of β -amyloid, tau protein deposits in APP/PS1 + ovCYS was decreased in DG, CA1, and CA3 regions compared with the APP/PS1 control. Moreover, the percentage of positive immunoreactive areas of β -amyloid and tau protein in ovocystatin treated NCAR was decreased in DG as well compared with the NCAR control. Hence, the obtained results imply protective mechanisms of ovocystatin in neurodegenerative diseases, which appears to be consistent with earlier findings for cystatin C biological functions (for review see [21]). We speculate that ovocystatin, due to its similarity to cystatin C [28], might induce protective pathways and prevent brain damage and neurodegeneration as well. There is convincing evidence that cystatin C plays an important role in aging and Alzheimer's disease [17]. First of all, cystatin C co-deposits with A β [21], binds to both A β _{1–42} and A β _{1–40} and inhibits A β fibril formation [23].

Indeed, some in vivo reports revealed that cystatin C association with A β inhibits A β oligomerization [24,44]. Additionally, higher cystatin C expression diminishes A β deposition [45,46]. Secondly, it has been suggested that cystatin C is endocytosed by damaged neurons and targeted to the lysosome, which results in inhibition of some lysosomal proteases and protects the cell from excessive lysosomal activity dysfunction [47]. Moreover, Sun et al. [48] demonstrated that cystatin C can prevent the inhibitory mechanisms of Cathepsin B-mediated A β degradation. Tizon et al. [44] also demonstrated the direct protection of neuronal cells from A β toxicity and induced apoptotic cell death by cystatin C. Neuroinflammation is currently recognized as an important pathophysiological feature of AD [49]. Sustained activation of brain-resident microglia and other immune cells (also peripheral lymphocytes), has been shown to exacerbate both amyloid and tau pathology. It leads to the development of the permanent inflammatory process resulting in apoptosis of neurons in the brain. Hence, the ability to modulate the inflammatory response is an important therapeutic aspect of AD. Unfortunately, our in vitro studies using bone-marrow-derived macrophages BMDM and primary mouse microglia excluded the potential immunoregulatory effect of ovocystatin (unpublished data).

In Alzheimer's disease, neuronal autophagy can also be impaired. This dysfunction may block the neuroprotective effects of autophagy, promote neuronal cell death and apoptosis and lead to the accumulation of toxic proteins, such as the tau protein [50]. Cystatin C may restore full functional autophagy via the mTOR pathway, which can be crucial for cell adaptation and survival under extreme conditions [51]. It has to be noted that our findings are consistent with the above-mentioned result because we found not only a decrease in A β but also decreased tau protein levels in hippocampus areas. Nevertheless, a recently published study by Duan et al. [52] revealed that cystatin C inhibits turnover of GSK3 β and promotes GSK3 β -catalyzed tau phosphorylation. Intraneuronal lysosomal/autophagosomal pathology observed in APP/PS1 mice is believed to be connected to the proteolytic processing of APP and tau protein, leading to the generation of toxic carboxy-terminal fragments and oligomeric β -amyloid and truncated forms of tau protein [53]. Thus, compromising lysosomal proteolytic activity by cystatin (i.e., cathepsin B, L, H, and asparaginyl endopeptidase) may prevent the enhanced production of invalid APP and tau fragments. This is another indication that cystatin C plays a protective role via APP-stimulated increase in cystatin C secretion, which mediates neural stem/progenitor cells [54]. Moreover, cystatin C interaction with fibroblast growth factor 2 (FGF-2) could lead to neurogenesis stimulation in the dentate gyrus in the rat's hippocampus. Regarding the current interesting reports about the role of *Porphyromonas gingivalis* in the course of Alzheimer's disease [6], it should be noted that both cystatin C and ovocystatin have antibacterial activity, including *Porphyromonas gingivalis* [55,56]. Hence, the exact role of cystatin C and ovocystatin in neuropathogenesis remains unclear and needs further studies.

However, this study has certain limitations that should be discussed. First, our sample was relatively small due to recommendations for using animals for research purposes. Second, the APP/PS1 transgenic mice are a model referring to the sporadic form of Alzheimer's disease. Additionally, the effect of gender on the mechanisms of action, which is still not sufficiently explained, could not be assessed due to the fact that only male mice were enrolled in the study. Another point is that we applied ovocystatin only in one dose (40 µg/mouse) based on previously published studies by Stańczykiewicz et al. [33]. Moreover, the evaluation was only done after intraperitoneal administration, while the foregoing data suggest that oral supplementation has therapeutic properties. Hence, further studies using oral administration are warranted. It should also be noted that, similarly to our previous results [32,33] we have not assessed the influence of ovocystatin on the expression of endogenous mouse cystatin C [57]. Numerous lines of studies revealed that the inhibitor E64d is an excellent tool compound for preclinical testing [58], and significantly improves memory and reduces Aβ [59]. To the best of our knowledge, only two studies revealed the beneficial effect of in vivo cystatin C administration on the neurodegeneration process. Namely, Nagai and colleagues [60] revealed that β-amyloid was not deposited in the hippocampus following cystatin C administration, which could support our hypothesis that exogenous ovocystatin inhibits β-amyloid oligomerization and deposition and may reduce cognitive deterioration, as we previously discussed [36]. Moreover, recently published results by Watanabe et al. (2018) showed that intraventricular administration of Cys C demonstrates a neuroprotective effect in other neurodegenerative diseases, i.e., amyotrophic lateral sclerosis (ALS), and suggest that Cys C may represent a novel therapeutic candidate for ALS [61]. Taken together, all these results indicate that cystatin C and other inhibitors such as E64 reduce β-amyloid and might play a pivotal role in neuroprotection. Thus, using other inhibitors as controls, such as cystatin C and general inhibitors of cysteine peptidases E64, would significantly improve the value of our results. These limitations should be addressed in further studies on ovocystatin's mechanism of action in the CNS.

5. Conclusions

Findings from our study point to the hypothesis that the administration of ovocystatin may not only reduce memory impairment in APP/PS1 transgenic mice, as was shown previously, but might also be a useful agent against Aβ oligomerization and consequent amyloid fibril formation and tau protein deposition. However, to confirm this hypothesis, more morphological, biochemical, and immunohistochemical analyses are needed. Further pharmacokinetic, stability, and distribution studies are necessary to assess its potential therapeutic properties.

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Data Availability Statement: The data analyzed during this study are included in this published article. Further inquiries can be directed to the corresponding authors.

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Article

Effect of Ovocystatin on Amyloid β 1-42 Aggregation—In Vitro Studies

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Abstract: Amyloid β peptides ($A\beta$) aggregating in the brain have a potential neurotoxic effect and are believed to be a major cause of Alzheimer's disease (AD) development. Thus, inhibiting amyloid polypeptide aggregation seems to be a promising approach to the therapy and prevention of this neurodegenerative disease. The research presented here is directed at the determination of the inhibitory activity of ovocystatin, the cysteine protease inhibitor isolated from egg white, on $A\beta$ 42 fibril genesis in vitro. Thioflavin-T (ThT) assays, which determine the degree of aggregation of amyloid peptides based on fluorescence measurement, circular dichroism spectroscopy (CD), and transmission electron microscopy (TEM) have been used to assess the inhibition of amyloid fibril formation by ovocystatin. Amyloid beta 42 oligomer toxicity was measured using the MTT test. The results have shown that ovocystatin possesses $A\beta$ 42 anti-aggregation activity and inhibits $A\beta$ 42 oligomer toxicity in PC12 cells. The results of this work may help in the development of potential substances able to prevent or delay the process of beta-amyloid aggregation—one of the main reasons for Alzheimer's disease.

Keywords: ovocystatin; amyloid beta peptide; ThT; CD; TEM; Alzheimer's disease



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1. Introduction

Neurodegeneration is a progressive process of neurons' destruction that leads to their death, entailing, e.g., abnormalities in signal transduction pathways controlling neuronal functions. At the cellular level, neurodegenerative processes are promoted by oxidative stress [1–3], mitochondrial dysfunction [4], trophic factor deficiency [5,6], and excessive secretion of pro-inflammatory mediators [7]. In the development of neurodegenerative diseases, one of the main risk factors is aging, which, with genetic and environmental factors, leads to the manifestation of the illness. The scientific literature has presented many mechanisms contributing to Alzheimer's disease (AD), including amyloid- β ($A\beta$), protein tau, and apolipoprotein E (APOE), as the crucial elements of the AD pathophysiology [8]. It has been extensively demonstrated that mutations in amyloid precursor protein (APP) and presenilin 1 (PSEN1) and 2 (PSEN2) genes lead to the development of AD and the production of toxic $A\beta$ peptides [9,10]. Thus, the deposition of amyloid beta 1–42 ($A\beta$ 42) is one of the most important pathological factors in AD, resistant to the action of proteolytic enzymes, showing toxic effects by inducing inflammation and finally neuronal cell death [11]. It has been shown that soluble $A\beta$ species, mainly $A\beta$ 42 oligomers, exert a pivotal role in the pathogenesis of synaptic damage, especially in the early stage of AD [12].

It has also been established that A β —induced neurotoxicity occurs mainly through the induction of apoptotic pathways in neurons [13,14].

Many studies have demonstrated that cysteine proteases constitute an important factor in neurogenesis as well as in the pathology of neurodegenerative disorders. Indeed, cysteine protease inhibitor—cystatin C (Cys C)—is implicated in neuroprotection and repair in the nervous system in response to diverse neurotoxic conditions [15]. It has been widely indicated that Cys C plays a pivotal biological role in Alzheimer's disease [16]. Firstly, Cys C co-deposits with A β plaques in patients with AD brains. Additionally, a specific, saturable, and high-affinity binding between Cys C and both A β 42 and A β 40 has been determined [17]. Secondly, it has been reported that Cys C possesses inhibitory properties against amyloid fibril formation and oligomerization [18]. Additionally, Mi et al. suggested that endogenous Cys C is a carrier of soluble A β in the brain, blood, and cerebrospinal fluid (CSF), where it inhibits A β aggregation into insoluble plaques [19]. Finally, neuroprotective roles of Cys C, including the inhibition of cysteine proteases, such as cathepsins B, H, K, L, and S, the induction of autophagy, and regulation of cell proliferation, have been demonstrated [16,20].

Considering these findings, it becomes increasingly important to develop new therapeutic strategies, such as nutraceutical drugs, which are potentially neuroprotective. Indeed, bioactive peptides/proteins are promising candidates for use as the inhibitors of A β fibril formation, such as ovocystatin (ovoC). Interestingly, ovocystatin improves cognitive function in young rats [21] and prevents aging-related cognitive impairment in older animals [22]. Moreover, recently published studies showed that the administration of ovoC in drinking water might be effective in the prevention of cognitive deterioration in APP/PS1 mice [23]. Additionally, ovoC could also be applied as a useful factor against A β oligomerization and consequent amyloid fibril formation and tau protein deposition [24]. OvoC is a small protein inhibitor of cysteine proteinases and the best model protein under study representing the cystatin superfamily [25]. Similar to Cys C, ovoC inhibits the lysosomal cathepsins, such as cathepsin B, H, K, L, and S [26], and it has comparable biological properties [27]. Moreover, it is highly homologous to human Cys C (62% structural similarity). Nevertheless, there is a lack of indication of the crucial properties of ovoC and its mechanisms of action, related to the pathology of aging-associated neurodegeneration.

Based on our animal model research results, this study aimed to assess the effect and mechanism of ovoC as an inhibitor of A β 42 fibrillation. Our findings provide an important insight into a new potential anti-neurodegenerative approach for an efficient inhibitor that can reduce the intensity of A β 42 fibril formation and grounds for developing adjuvant treatment strategies based on nutraceuticals.

2. Results

2.1. Isolation and Purification of Ovocystatin

The preparation of ovoC from 20 eggs typically yielded 17–18 mg of the pure inhibitor with excellent biological activity ranging in U/mg between 23 and 25 as assessed using Barrett's colorimetric method. The isolation and lyophilization conditions did not induce polymerization of ovoC and allowed us to obtain homogenous preparation of inhibitor. The isolation and lyophilization conditions did not induce polymerization of ovoC and allowed us to obtain homogenous preparation of inhibitor. The molecular weight of the obtained protein is almost the same migration distance of the 14 kDa molecular weight marker and agrees with the chicken egg white cystatin molecular weight of approximately 13 kDa (Figure 1). The purity of ovoC determined using HPLC was greater than 95% (Figure 2).

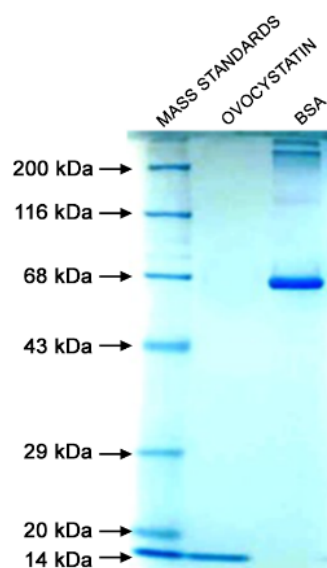


Figure 1. PAGE-SDS electrophoresis of preparation of ovocystatin in 10% gel (reducing conditions). The lanes were loaded according to the order (from left): 10 μ L of the mass standards (Protein Marker III), 10 μ L of 0.2 mg/mL ovocystatin, 10 μ L of 0.2 mg/mL BSA, and separation was carried out in 25 mM TRIS/192 mM glycine buffer pH 8.3 with 0.1% SDS for 90 min. The bovine serum albumin was loaded as an additional control.

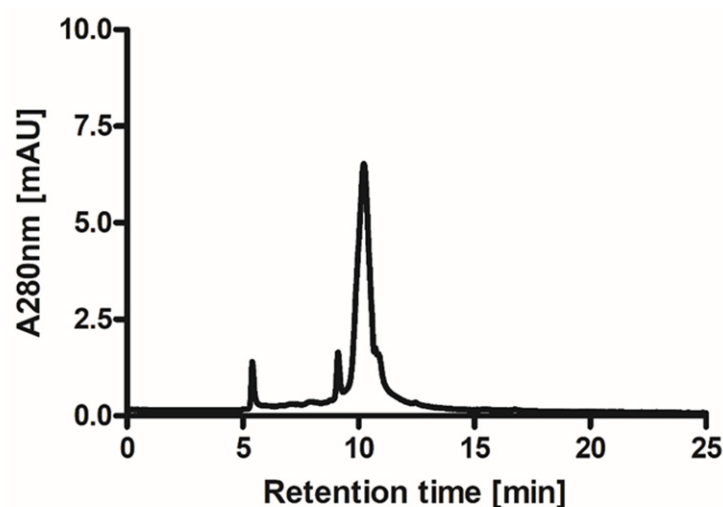


Figure 2. HPLC gel filtration is the final ovocystatin preparation. Twenty microliters of the sample were loaded on the Bio SEC-5 column and eluted with 150 mmol/L sodium phosphate, pH 7.4. Protein in the eluent was detected by UV absorption measurement at 280 nm. The retention time determined for ovocystatin was 10.2 min.

2.2. Effects of Ovocystatin against A β 42 Fibrils Formation by ThT Assay

To explore if ovoC exerted an inhibitory effect on A β 42 fibril formation, the ThT assay was employed (Figure 3). ThT selectively binds to the aggregated β -sheet fibrils of A β structures and leads to a significant increase in fluorescence of ThT proportional to the amount of the amyloid fibrils formed [28]. As can be seen in Figure 3a, under our experimental conditions (pH 7.4 and 37 $^{\circ}$ C), ovoC alone did not form fibrils; however, the A β 42 solution presented a typical sigmoidal curve [29,30]. With the increasing incubation time, an augmentation in the number of fibrils was observed with the half-time ($t_{1/2}$) of aggregation at 5.83 h (Figure 3c–e). The curve reached the plateau after 16 h, indicating the completion of amyloid fibril formation.

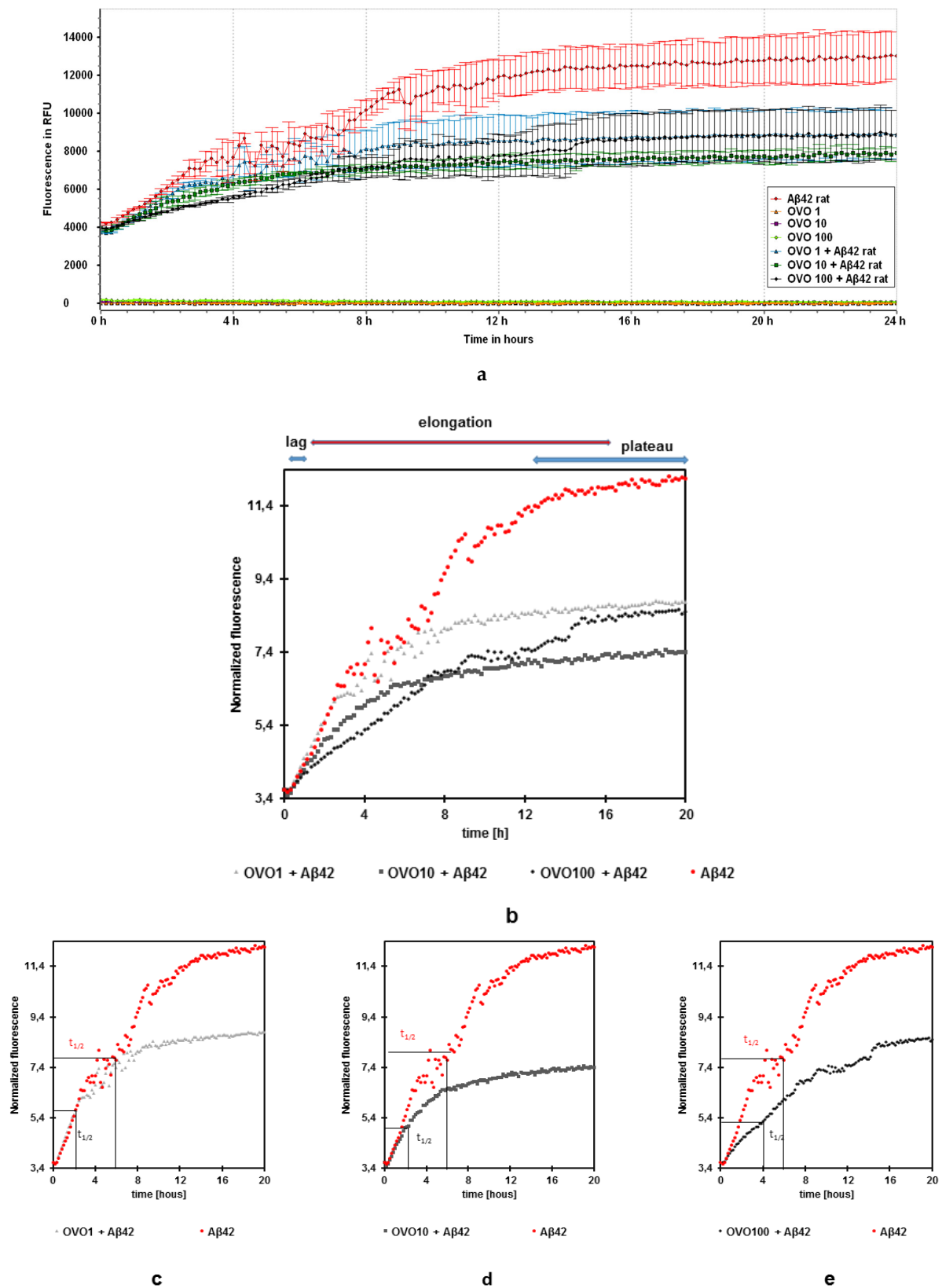


Figure 3. Efficacy of ovocystatin against Aβ42 aggregation. Aβ42 fibrils formation without or with ovocystatin (ovo) was monitored using ThT fluorescence assay. Fluorescence intensity was measured at an excitation wavelength of 420 nm and an emission wavelength of 480 nm (a). Normalized fluorescence curves with lag, elongation, and plateau phases (b), and half time of aggregation of Aβ42 (20 μM) alone, (c) Aβ42 with ovo 1 μg/mL, (d) Aβ42 with ovo 10 μg/mL, and (e) Aβ42 with ovo 100 μg/mL.

The kinetics of disaggregation of A β 42 by ovoC are shown in Figure 3a (fluorescence in RFU) and Figure 3b (normalized fluorescence). When ovoC was incubated with A β 42 solution, the fluorescence intensity at 480 nm decreased substantially, which indicated that the reduction in A β 42 fibrillogenesis takes place. The changes are observed mainly in the elongation phase. Assuming that the A β 42 plateau value is 100% of aggregation (RFU = 13,013), in the presence of ovoC, the level of amyloid fibrils was reduced to 68.7% for 1 μ g/mL, 60.7% for 10 μ g/mL, and 68.4% for 100 μ g/mL of ovoC. The half-time of aggregation ($t_{1/2}$) defined here as the time to reach half the maximum fluorescence intensity, varies depending on the ovoC concentration. With the increase in ovoC concentration, the time $t_{1/2}$ increases, and the kinetics of aggregation changes (2.17 h for 1 μ g/mL (Figure 3c), and 10 μ g/mL (Figure 3d) of ovoC, and 4 h for 100 μ g/mL of ovoC (Figure 3e)). The elongation curves for A β 42 and ovoC (1 and 10 μ g/mL) + A β 42 almost overlap in the initial phase of elongation; however, the addition of ovoC causes a decrease in the possible level of saturation, and thus a decrease in the final fluorescence and shortening of the $t_{1/2}$. However, the log phase of studied samples was also slightly lower when compared to A β 42 alone, which may suggest the possibility of ovoC interaction with monomers, dimers, or oligomers. Thus, averaging the observations obtained from the three experiments, it is visible that ovocystatin reduces the aggregation of the A β 42 peptides.

2.3. Effects of Ovocystatin on A β 42 Fibril Morphology

To confirm the observed inhibitory effect of ovoC on A β 42 aggregation, TEM was employed to visualize changes in fibril morphology. A β 42 alone exhibited a large amount of long and thick amyloid fibrils creating large clusters after 48 h of incubation at 37 °C (Figure 4a,b). OvoC, which showed inhibitory activity in the ThT assay, significantly reduces A β 42 fibril density and length, compared to the A β 42 alone (Figure 4c,d). The highest concentration of ovoC: 100 μ g/mL reduced the amounts of amyloid aggregates most effectively (Figure 4d). These results support the effects of *ovocystatin* against A β 42 fibril formation by ThT assay.

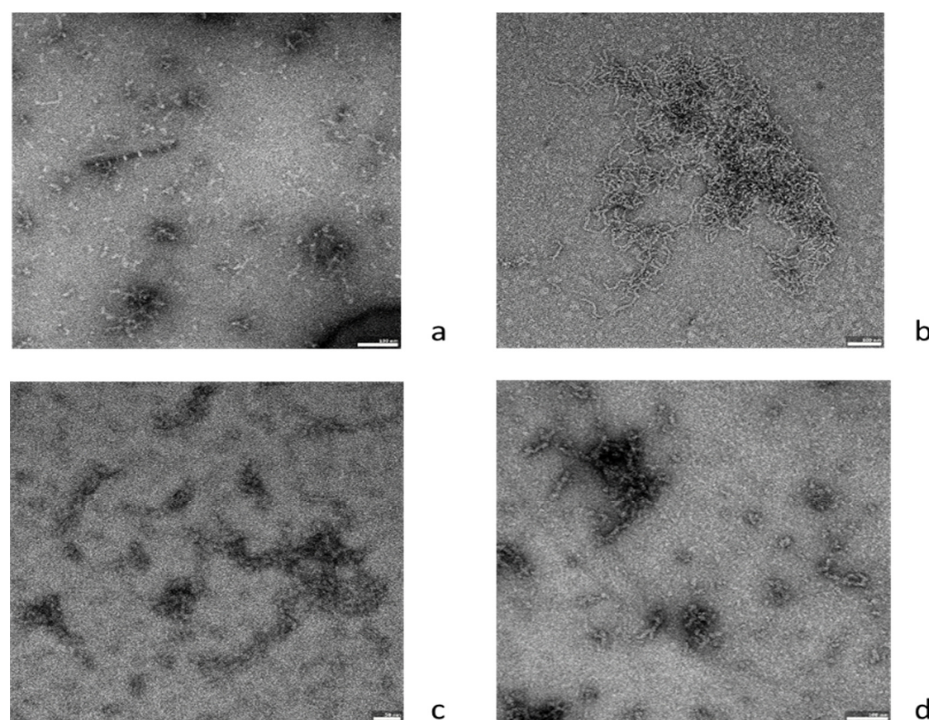


Figure 4. Representative transmission electron microscopy images of (a) A β 42 at $t = 0$ ($n = 43$), (b) A β 42 after 48 h incubation ($n = 41$), (c) A β 42 + ovoC (10 μ g/mL) after 48 h incubation ($n = 48$), and (d) A β 42 + ovoC (100 μ g/mL) after 48 h incubation ($n = 39$). Scale bars represent 0.5 μ m.

2.4. Effects of Ovocystatin on A β 42 Secondary Structure during Fibrillation

CD spectroscopy in the far UV region (190–240 nm) was used to estimate changes in the secondary structure of the A β 42 sample alone or incubated in the presence of ovoC. Using CD spectroscopy, the typical three phases process is measured, including a lag phase without significant conformation changes, an exponential phase including a rapid increase in β -sheet presence, and a plateau phase, in which the secondary structure of the β sheet dominates. The overlays of CD spectra of A β 42 alone and A β 42 + ovoC at concentrations of 1, 5, and 10 μ g/mL are presented in Figure 5. As can be seen in Figure 5a, we observed the transition from the non-amyloidogenic unordered/ α -helix of soluble A β 42 to the amyloidogenic β -sheet conformation. After 5 h incubation, both A β 42 peptide and A β 42/ovoC mixture exhibited a strong positive band at 218 nm, suggesting the conformational changes of A β 42 from α -helix to β -sheet state (IC₅₀: 6.105, 5.621, 5.237 and 5.291 for A β 42 alone, A β 42 + ovoC 1 μ g/mL, A β 42 + ovoC 5 μ g/mL and A β 42+ovoC10 μ g/mL, respectively). β -sheet secondary structure formation after 16 h is indicated by the appearance of a negative band at 218 nm and a positive band at 200 nm. For the A β 42 sample treated with ovoC, no differences in the structural transition of A β 42 were observed (Figure 5b–d) compared to those observed in the A β 42 incubated alone (Figure 5a). These observations suggested that ovoC did not affect the intensity of the A β 42 aggregates' transformation from the initial structure to the β -sheet protein.

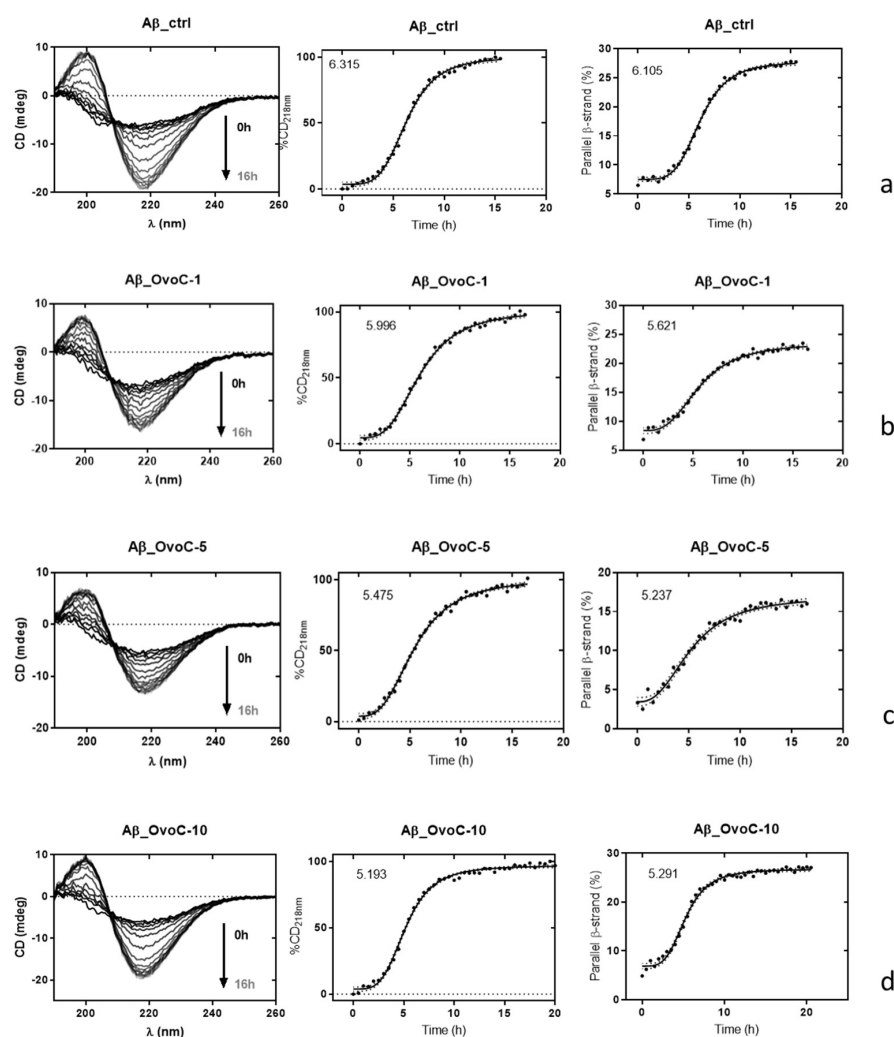


Figure 5. Circular dichroism spectra of amyloid β 42 (A β) in the absence (a) and in the presence of ovocystatin at 1 μ g/mL (b), 5 μ g/mL (c), and 10 μ g/mL (d) concentration. Ctrl—control; amyloid β alone—A β ; ovoC—ovocystatin; amyloid β 42 + ovocystatin: A β + ovoC.

2.5. Ovocystatin Increased PC12 Cells Viability

To determine if ovoC (10 and 100 $\mu\text{g/mL}$) and A β 42 affect PC12 cell viability, an MTT assay was performed. One-way ANOVA analysis revealed a 10% ($\text{OD}_{570} = 0.783$) increase in PC12 cell proliferation of ovoC (10 $\mu\text{g/mL}$)-treated PC12 cells, while ovoC (100 $\mu\text{g/mL}$) showed an effect comparable to the control cells ($\text{OD}_{570} = 0.708$) but is not toxic to cells (Figure 6a). A β 42 reduced PC12 cell viability to 85% ($\text{OD}_{570} = 0.608$). To determine whether ovoC can inhibit A β 42-dependent toxicity, A β 42 (10 μM) with and without ovoC (10 and 100 $\mu\text{g/mL}$) was added to PC12 cells for 24 h. An increase in PC12 cell viability was observed when A β 42 was applied together with ovoC. Cell survival increased to 105% for ovoC (10 $\mu\text{g/mL}$) ($\text{OD}_{570} = 0.743$) and to 101% for ovoC (100 $\mu\text{g/mL}$) ($\text{OD}_{570} = 0.717$) compared to cells treated with A β 42 alone (Figure 6b). Thus, this data indicate that ovocystatin increased the viability of A β 42-treated cells.

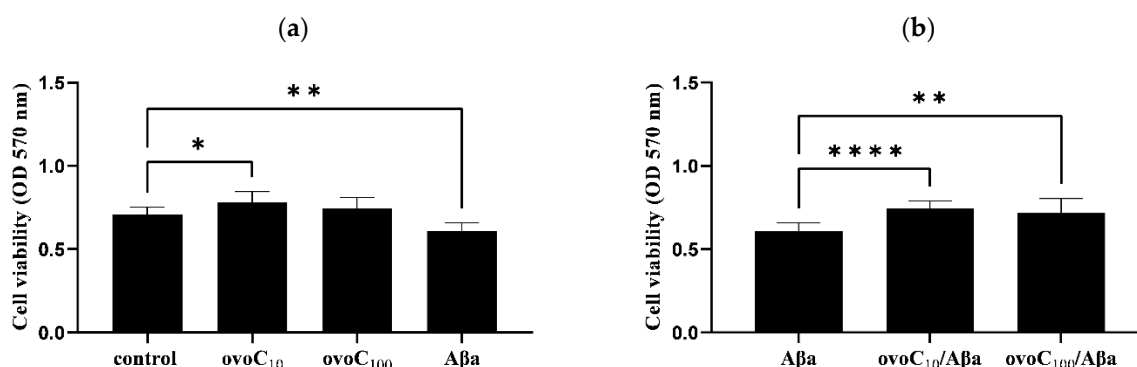


Figure 6. Viability of PC12 cells treated with A β 1–40 (20 μM) alone (a) or with different concentrations of ovoC (b) for 24 h. Cell viability was measured with MTT assays and is shown as a percentage of the untreated cells (control). Data were analyzed by one-way ANOVA to evaluate treatment effect. * $p \leq 0.038$ and ** $p \leq 0.002$ when compared with control cells. **** $p \leq 0.0001$ and ** $p \leq 0.01$ when compared with A β 42 alone.

3. Discussion

Alzheimer's disease is the most frequent cause of dementia in the elderly. Unfortunately, there is no effective treatment able to prevent or stop AD. Up until now, clinical trials have focused mainly on patients who have developed symptoms. However, the importance of using therapies that could prevent the development of pathological changes in the brain has presently been highlighted [31]. The accumulation of insoluble amyloid β deposits forming the senile plaques is observed in the brain and is a hallmark of AD. Fibrillation proceeds via oligomers to protofibrils and fibrils of A β 42 leading finally to the production of the insoluble and toxic senile plaques. Aggregated A β mainly takes the form of β -sheet conformation. This form seems to be extremely toxic to the neurons [32].

Biologically active substances, safe, bioavailable, and exhibiting neuroprotective potential may constitute an important therapeutic aspect in preventing and/or inhibiting the development of neurodegenerative diseases. One of them can be cystatin isolated from chicken egg white, named ovocystatin, which could be widely used in medical research due to its structural and biological similarities to human cystatin C possessing a beneficial effect on the inhibition of cysteine proteases and A β aggregation [19,33]. The ovocystatin preparation method developed by us allows us to obtain a pure ovocystatin preparation, without additional ingredients and impurities. The buffers containing volatile salts were used, and were removed during the lyophilization process. This preparation procedure makes it possible to obtain a monomeric form of ovocystatin with very high activity and purity reaching nearly 100%. Moreover, the inhibitor isolated by this method has already been tested in some studies, where its purity and cytotoxicity were also checked [34,35]. The results obtained indicate that our ovocystatin preparation is not contaminated with any substances.

It has been shown up until now, that ovocystatin has a beneficial impact on cognitive function in young rats, and might prevent aging-related cognitive impairment in older animals [21] and reduce memory decline in APP/PS1 mice model [23,24]. However, its potential molecular mechanism of action is not explained and needs to be examined in detail. It was determined by us in our latest paper that ovoC induced changes in the expression of Alzheimer's disease—A β and tau proteins in APP/PS1 mice model [24]. Therefore, in this study, we focused our attention on examining the ability of ovoC to interact with A β 42, inhibit fibrils, or control oligomer growth into non-toxic species. To follow the amyloid fibrils' growth ThT fluorescence assay, TEM and CD were used. In ThT assay growth of fluorescence is the result of ThT binding to any β -sheet of amyloid, therefore studying this phenomenon does not provide information on the length of the resulting fibers but only on the total number of fibrillar structures [32,36]. Figure 3 shows, that after 24 h of incubation of A β 42 at 37 °C, aggregation occurred in the form of a sigmoidal curve, in agreement with previous studies [29,30,37,38]. OvoC, at 1–100 μ g/mL concentration could reduce amyloid fibril growth, picking at 10 μ g/mL (39.3% inhibition). A total of 1 μ g/mL and 100 μ g/mL of ovoC reduced fibril formation up to 68.2%, and 68.4%, respectively, compared to A β alone taken as 100%. Additionally, the time $t_{1/2}$ increases, and the kinetics of aggregation change with ovoC concentration (Figure 3c–e). The elongation curves for A β 42 and ovoC (1 and 10 μ g/mL) + A β 42 almost overlap in the initial phase of elongation, however, the addition of ovoC causes a decrease in the possible level of saturation, and thus, a decrease in the final fluorescence and shortening of the $t_{1/2}$. These observations indicate that ovoC may have anti-aggregative activity (especially at lower doses), contributing to PC12 increased viability, as was noticeable in the MTT assay (Figure 6). Similar activity was observed in other preparations, e.g., PRP complex [39,40], ginseng [41], baicalein [42], or tucarecol [43].

To provide more insight into the anti-fibrillation activity of ovoC, TEM observation was conducted to evaluate fibrillary morphology. As was shown, after 24 h of incubation, abundant amorphous and disordered aggregates were presented in the A β 42 sample, and the aggregation process intensified significantly after 48 h of incubation (Figure 4a,b). However, TEM images of A β 42 incubated with ovocystatin indicated the reduction in the A β fibril density and length (Figure 4c,d), compared with A β alone (Figure 4b). This means that ovoC can inhibit A β fibrillation. The images obtained by TEM confirmed our observation in the ThT fluorescence test.

Furthermore, CD spectroscopy was performed to explore the impact of ovocystatin on the A β 42 peptide secondary structure. Figure 5 presents the changes in CD signals along with the varying amounts of ovoC added to A β 42. Unfortunately, the results presented indicate that ovoC did not change the β -sheet secondary structure of A β 42 with the lapse of time, which precludes ovocystatin potential action as a breaker of the amyloid β structure. Thus, the inhibition of amyloid aggregation observed in ThT may occur without affecting the changes in the β -sheet secondary structure of A β 42. The ThT, TEM, and CD analysis data led us to propose the potential mechanism of ovoC action. Because changes were mainly observed in elongation phase, and slightly in the A β 42 log phase (nucleation phase), the possibility of ovoC interaction with monomeric A β leading to inhibited seeding process is considered. However, it is more plausible that ovoC can stabilize the oligomers and/or protofibrils and slow down their conversion to fibrils. Such activity was also observed for human cystatin C [33,44] and some polyphenols, including, among others, resveratrol [45,46]. It was also demonstrated in the literature, that A β fibril formation can be controlled by specific amino acids within the A β peptide itself, and various (still not fully known and intensively studied) A β peptide regions contribute differently to A β aggregation and have identified crucial interactions among specific peptide regions controlling this process [47]. So, we hypothesize, that another possibility is to target, with ovoC, a specific subregion of A β 42, blocking by this way the interactions between monomers and oligomers, thereby preventing the formation of further fibrils and aggregates.

OvoC was built up of a five-stranded anti-parallel β -sheet wrapping around a central α -helix. The linkage within the β -sheet is (N)- β 1-(a)- β 2-L1- β 3-(AS)- β 4-L2- β 5-(C). As is a broad 'appending structure' located beyond the compact core of the molecule. It is probable that the interaction between ovoC and A β takes place via the C-terminal hydrophobic sequence (sequence: VYSIPWLNQIKLLESKC; L2- β 5; aa 99–115). A similar interaction was reported for human cystatin C (sequence: IYAVPWQGTMTLSKS; L2- β 5; aa 101–117). This region showed the highest inhibitory effect toward A β -fibril formation. The association of human cystatin C and A β was specific, saturable, and of high affinity. This region is located in the C-terminal part within the L2 loop and β 5 strand of the protein that are exposed to the environment. The C-terminal epitope mediates the interaction of A β with the L2- β 5 part without any restriction [48,49]. Human cystatin C is highly homologous to chicken ovoC (62% structural similarity) and shares similar biological properties [26]. Therefore, the results from studies on human cystatin C might be strongly supportive of interpreting the molecular mechanisms of ovoC interactions with other proteins [27]. In order to confirm the above hypotheses, it is necessary to conduct further studies with the use of selective negative controls, for example, low molecular weight compounds that mask this site or missense mutants that lack this region.

Numerous literature reports of experimental studies have focused on the effect of naturally occurring substances on self-aggregated A β peptides. In particular, products with minimal side effects with the potential ability to pass the blood–brain barrier (BBB) are of particular interest [45,46,50]. Alghazwi et al., [51] determined the inhibitory effects of *Ecklonia radiata* fractions on A β 42 amyloid fibrillation. It was also shown that polyphenolic-rich fucoidan samples isolated from *Fucus vesiculosus* possess high anti-aggregative activity [52]. Moreover, curcumin, one of the most common polyphenolic compounds, possesses comparable properties [53]. Molecular docking and dynamic studies demonstrated that natural compounds could bind A β 25–35 and could break the peptide leading to losing a significant quantum of β -sheet content resulting in inhibiting A β 25–35 aggregation [54].

The results obtained from the ThT assay, TEM, and CD suggested that ovocystatin inhibits the elongation phase of A β 42 aggregation breaking A β 42 fibrillation, which points to a more relevant role in the amyloid morphology changes. This study highlights the potential of ovocystatin as a promising neuroprotective compound that has anti-aggregation effects on A β 42 formation. In the next studies, we will try to explain the inhibitory and disaggregation mechanisms of ovocystatin more specifically. Furthermore, it is crucial to examine its cytoprotective activity using cell lines as in vitro models and to study the preventive effect on cognitive function using transgenic animals as an in vivo experiment.

4. Materials and Methods

4.1. Materials

1,1,1,3,3,3-Hexafluoro-2-propanol (99%) (HFIP), Thioflavin T (ThT), papain from papaya latex, N_{α} -Benzoyl-DL-arginine β -naphthylamide hydrochloride (BANA), Tris(hydroxymethyl)aminomethane (TRIS); glycine; N,N,N',N'-Tetramethyl ethylenediamine (TEMED), sodium dodecyl sulfate (SDS), and bovine serum albumin (BSA) were purchased from Sigma (Saint Louis, MO, USA). Amyloid β 42 (A β 42) was obtained from Tocris (Bristol, UK). Protein Marker III was purchased from AppliChem (Darmstadt, Germany). SimplyBlue Safe Stain was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

4.2. Ovocystatin Isolation

Ovocystatin was prepared from chicken egg white according to a method based on affinity chromatography with immobilized S-carboxymethylated papain as described by Anastasi et al. [55] with slight modifications [56] and dialyzed overnight against 50 mM ammonium bicarbonate with several changes of the dialysis buffer. The volume of the preparation and the protein concentration was measured to calculate the content of the inhibitor. The protein concentration was estimated using an extinction coefficient at 280 nm

equal to 0.871 for 0.1% ovocystatin solution. The final preparation was aliquoted and lyophilized for long-term storage. The quality of the inhibitor was assessed concerning the antipapain activity, against N_α -Benzoyl-DL-arginine β -naphthylamide (BANA) [57], and the electrophoretic distribution in SDS-PAGE using 10% resolving gel in reducing conditions [58]. The proteins were visualized with colloidal Coomassie SimplyBlue SafeStain. Gel filtration chromatography on an HPLC system (Agilent 1100, Agilent Technologies) was used for the determination of ovocystatin purity. Twenty microliters of the sample were loaded on a Bio SEC-5 column (300.0×7.8 mm secured with a Bio SEC-5 guard column (50.0×7.8 mm) and eluted with 150 mmol/L sodium phosphate, pH 7.4 at the flow rate 1.2 mL/min. The protein in the eluent was detected by UV absorption measurement at 280 nm.

4.3. Amyloid β 42 Preparation for Thioflavin T (ThT) Assay and Transmission Electron Microscopy (TEM)

A β 42 lyophilized powder was monomerized by a 20 min pretreatment with 1,1,1,3,3,3-hexafluoro-propanol (HFIP) at RT. The solution was then sonicated for 15 min, and finally, the solution was evaporated overnight at RT. The thin film obtained was dissolved in 0.1% $\text{NH}_4\text{OH}/\text{H}_2\text{O}$ and sonicated for 5 min in an ice bath. Finally, the 250 μM stock solution obtained was immediately diluted to the required concentration for an experiment.

4.4. ThT Assay of A β 42 Fibril Formation

To determine the amyloidogenic structure of A β 42 Thioflavin T (ThT) assay was performed. This is a dye that exhibits a strong increase in its fluorescence during binding to the β -sheet structure of A β , thus enabling the quantification of the presence of fibrous species. ThT was dissolved in a sterile phosphate buffer (100 mM, pH 7.4) to a final concentration of 10 μM and vortex 10 min at RT. A β 42 (10 μM) fibrillation was measured with or without the presence of ovoC studied at concentrations of 10, and 100 $\mu\text{g}/\text{mL}$, using 96-well black plates. ThT was added to the reaction mixture just before starting the measurement. The final volume of the samples was 100 μL . Kinetics fluorescent data were collected at 37 $^\circ\text{C}$ in triplicate using CLARIOstar[®] Microplate Reader (BMG LabTech, Offenburg, Germany), with measurements acquired at 15 min intervals. The excitation and emission wavelengths were set at 440 and 480 nm, respectively. The experiment was repeated twice in three independent repetitions ($n = 6$). Pure ThT solution was used as a blank to overcome the autofluorescence issue. The single measurement point presented on the plot is based on an average value of three independent repetitions, and the error bars represent their standard deviation.

4.5. TEM

Samples: A β 42 alone (10 μM), A β 42 (10 μM) + ovoC (10 $\mu\text{g}/\text{mL}$), or A β 42 (10 μM) + ovoC (100 $\mu\text{g}/\text{mL}$) were incubated for 0, 24, and 48 h at 37 $^\circ\text{C}$. The final volume of the samples was 100 μL . The samples were centrifuged (5 min, 50 μg), and the obtained pellets were fixed in 2.5% glutaraldehyde (POCH) for 24 h. Next, the samples were centrifuged again (5 min, 50 μg). A total of 10 μL of the sample was placed on copper grids (400 Mesh) with formvar film and carbon coating (Agar Scientific, Stansted, UK). Prepared samples were contrasted and performed with 2% uranyl acetate (MicroShop, Piaseczno, Poland). Imaging was performed using a JEOL 1200 microscope (Peabody, MA, USA) JEOL Japan microscope.

4.6. Circular Dichroism Spectroscopy (CD)

4.6.1. A β 42 Preparation for CD Measurement

A β 42 lyophilized powder was monomerized by a 20 min pretreatment with 1,1,1,3,3,3-hexafluoro-propanol (HFIP). The solution was then sonicated in an ice bath for 15 min, and finally, the solvent was evaporated overnight at RT. The resulting thin film was dissolved in NaOH (10 mM). A total of 20 μL aliquot containing 41.7 μg A β 42 was diluted using 180 μL

phosphate buffer (10 mM) that contained NaCl (10 mM), pH 7.4. The final solution for CD measurement contained 200 μ L of A β 42 at a concentration of 47.1 μ M at pH 7.6.

4.6.2. CD

Samples: A β 42 alone, A β 42 + ovoC (1, 5, and 10 μ g/mL), were analyzed. Spectra were recorded on a J-1500 spectropolarimeter (Jasco, Japan) equipped with a thermostated cell holder and a PM-539 detector. CD spectra were recorded in the spectral range 190–260 nm using a 0.1 cm path length quartz cell at 37 °C. Spectra were accumulated six times. All values were corrected for solvent contributions—phosphate buffer (10 mM) that contained NaCl (10 mM), pH 7.6. Measurement conditions: data pitch, bandwidth, and D.I.T. were 0.5 nm, 1 nm, and 1 s, respectively, at 50 nm min^{−1}. In co-incubation experiments of A β 42, the ovoC spectrum was subtracted from the mixture spectrum to obtain only A β 42 CD contribution.

To follow conformational changes and for inhibition studies, the CD signal at 218 nm was plotted vs. the time of incubation. Additionally, we used the BeStSel web server, [59] which provides a method to analyze the CD spectra with detailed secondary structure information, including parallel and antiparallel sheets. Using the obtained data parallel, β -strand content (%) was plotted vs. time of incubation. Data analysis was performed using the GraphPad Prism 7.01 software (GraphPad Software Inc., San Diego, CA, USA).

4.7. Cell Viability Determination

4.7.1. Cell Culture

PC12 (Tet-On) cell line (ClonTech), a rat pheochromocytoma cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 5% horse serum, and penicillin-streptomycin (PS) at 37 °C, 5% CO₂ in a humidified incubator with the culture medium changed once every three days.

4.7.2. Amyloid Preparation for Cell Treatment

A β 42 lyophilized powder was monomerized by a 20 min pretreatment with 1,1,1,3,3,3-hexafluoro-propanol (HFIP) at RT. The solution was then sonicated for 15 min, and finally, the solution was evaporated overnight at RT. The thin film obtained was dissolved in DMSO. Finally, the 250 μ M stock solution obtained was immediately diluted in PBS to the required concentration for an experiment. To obtain oligomers A β 42 was incubated for 24 h at 37 °C, 5% CO₂ in a humidified incubator.

4.7.3. MTT Reduction Assay

Cell viability was determined by colorimetric MTT assay [60,61]. Cells were seeded onto a flat-bottomed 96-well culture plate (1 \times 10⁴ cells/well) and incubated for 24 h at 37 °C with ovoC preparation (10 and 100 μ g/mL) applied to the cells in the presence or absence of 24 h—aggregated A β 42 (10 μ M). After cell treatment, an MTT solution (5 mg/mL) was added, and the cells were incubated again for 4 h to develop formazan crystals. The formazan crystals were solubilized by adding 100 μ L DMSO and vigorously shaken to complete resolution. The absorbance was measured at 570 nm by an EnSpire™ 2300 microplate reader (Parkin Elmer, MA, USA).

4.8. Data Analysis and Graphical Visualization

Statistics and graphs were prepared using GraphPad Prism Software v9. Data are presented as mean \pm SD. Data were analyzed using the Ordinary one-way ANOVA test. A value of $p < 0.05$ or less is considered statistically significant.

5. Conclusions

The aggregation process of A β 42 monomers to potentially neurotoxic oligomers and fibrils seems responsible for the beginning of AD. The first stage of this process is connected with conformational changes to a β -sheet structure. Previous studies have shown

that ovocystatin improves cognitive function in young rats, prevents aging-related cognitive impairment in older animals, and induces changes in the expression of Alzheimer's disease—A β and tau proteins in the APP/PS1 mice model. In the present work, we presented the potential mechanism of action of ovocystatin isolated from egg white in vitro, on A β 42 fibrillogenesis using ThT fluorescence assay, transmission electron microscopy, and circular dichroism spectroscopy and tested cell viability by MTT assay. Our study has demonstrated that ovocystatin may reduce amyloid fibril formation and A β 42 aggregation, unfortunately without the ability to β -structure formation/destabilization. However, it was shown that ovocystatin reduced A β 42 toxicity in PC12 cells. Further studies will focus on explaining the potential mechanism of A β 42–ovocystatin interaction. It is also necessary to investigate whether the observed phenomenon will have a beneficial effect on the survival and functions of neurons. We hope that the results of this work may help in the development of an effective inhibitor able to prevent or delay the process of beta-amyloid aggregation.

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