**“Induction of autophagy as a mechanism for genistein action in experimental therapy of neurodegenerative diseases”**

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The term ‘neurodegenerative diseases’ is defined as diseases characterized by progressive loss of nerve cells [1]. They constitute a group of diseases with different causes (aggregation of macromolecules, demyelination, inflammation, increase in the level of reactive oxygen species), and their number is currently estimated at several hundred. Unfortunately, due to population aging, the incidence of these diseases is significantly increasing, and according to the World Health Organization (WHO), the number of people suffering from neurodegenerative diseases exceeds now almost 100 million worldwide [2, 3].

The reason for about 70% of neurodegenerative diseases is the aggregation of macromolecules in nerve cells which damages their proper functioning and leads to the occurrence of serious psycho-motor symptoms. Unfortunately, despite many studies on development of new drugs, up to now, they have failed to offer a therapy that would be effective in treating these diseases [2]. Pharmacological agents to alleviate their symptoms, rehabilitation or anti-depression therapy are used instead [3].

Currently, research is conducted on a number of therapeutic strategies which include inhibition of the expression of genes encoding pathogenic proteins, their binding and inactivation or inhibition of pathways leading to apoptosis [3, 4]. However, most of these strategies do not bring the expected results during the first phases of clinical trials or even during preclinical studies, while gene therapy, which would be effective in the case of genetic neurodegenerative diseases, seems to be still a long way off [5, 6].

Therefore, more attention is paid to the strategy of accelerated removal of accumulated pathological proteins from neurons. There are two major pathways of protein degradation in the cells: the proteasomal pathway and the lysosomal pathway in the autophagy process. Unfortunately, many literature data indicate serious disturbances in the functioning of the proteasome in the case of neurological diseases, which definitely limits the possibility of using this pathway of pathogenic factors’ degradation as a potential therapy [5]. For this reason, the autophagy process has been the center of attention of scientists working on the treatment of the described diseases. Autophagy is an evolutionary old, phylogenetically conserved process that occurs in all eukaryotic cells, and serves to degrade small cell organelles or long-lived, improperly functioning proteins. It is based on a membrane surrounding of macromolecules to be degraded and then enclosing them in a vesicle called autophagosome. Autophagosome is then fused with the lysosome to form autophagolysosom, and acidic lysosomal hydrolases digest the inside of the vesicle into monomers that can be again used by the cell. The whole molecular mechanism of the autophagy process, along with its regulation and potential of its use in the therapy of neurological diseases, was described by me in a review article published in *Metabolic Brain Disease* [7].

However, seraching for a compound that would not only induce autophagy but still be safe in long-term use (because patients will probably have to take it for the rest of their lives) and cross the blood-brain barrier (which is especially important in the treatment of diseases affecting the nervous system) is still going on. All these requirements turned out to be fulfilled by genistein, one of the flavonoids, which is particularly abundant in leguminous plants, mainly in soy beans. Genistein has a number of biological activities ranging from anti-tumor to anti-bacterial properties. The activation of the EB transcription factor (TFEB, also known as lysosomal biogenesis factor) by this isoflavone, suggesting induction of autophagy, which was discovered during the study on a rare genetic disease -

mucopolysaccharidosis (in which a completely different mechanism of action of genistein was used, namely inhibition of glycosaminoglycan synthesis as a result of negative regulation of the EGFR protein kinase activity - epidermal growth factor receptor [8, 9]), was a particularly important finding. Detailed studies on cellular and animal models of mucopolysaccharidosis, treated with genistein, provided information on its safety and the lack of adverse effects, even at very high doses, and crossing the blood-brain barrier [10, 11].

Considering the biological properties of genistein and the desired characteristics of a potential drug for diseases caused by protein aggregation, it was reasonable to test whether the action of genistein is effective in the potential treatment of this group of diseases affecting the central nervous system (CNS).

**Therefore, the purpose of my doctoral dissertation was:**

**(a) to examine effects of genistein on the efficiency of degradation of proteins that are the causes of two model neurodegenerative diseases;**

**b) to determine the molecular mechanism of genistein action;**

**(c) to investigate effects of genistein on behavioral changes in the animal model of the selected neurodegenerative disease.**

Two model neurodegenerative diseases were selected in my research:

a) Huntington's disease (HD) - as a model for a rare monogenic disease with well-established etiology and autosomal dominant inheritance;

b) Alzheimer's disease (AD) - as a model for the most common neurodegenerative disease, with an unknown etiology and multifactorial inheritance.

***Studies carried out on the Huntington's disease model***

HD is a disorder whose direct cause is a mutation consisting of the expansion of CAG nucleotide triplets in the 1st exon of the *IT15* (*HTT*) gene. In healthy people, the number of these repeats is less than 26, while HD patients usually have 40 or more CAG triplets (cases of 27-39 repeats form a transitional group). The increased number of CAG repeats results in a multiplication of the number of glutamine residues (polyQ chain) in the huntingtin protein (HTT), which prevents its correct folding. As a consequence, mutant huntingtin (mHTT) is deposited in cells in the form of insoluble aggregates, impairing their proper functions [12]. This results in the appearance of three types of symptoms: a) physical symptoms, such as sudden, uncontrolled, choreic movements, motor slowdown and dystonia; b) emotional symptoms such as depression, irritability, personality changes, obsessive-compulsive syndromes and aggression attacks; c) cognitive disorders, such as inability to make a decision, focus attention or difficulties in learning and remembering. The disease is progressive and leads to disability and death, most often due to swallowing disorders leading to aspiration pneumonia [12].

The only studies, performed previously and comprising genistein and HD, included the effects of this isoflavone on the rat HD model induced with 3-nitropropionic acid (3-NPA) [13, 14]. However, the use of this model does not allow the study on the primary cause of the disease, because 3-NPA does not induce the formation of mHTT in HD animals, and only mimics the secondary effects of the disease. Thus, the effect of genistein on mHTT has never been determined.

As a research model, I used HEK-293 cell line which was transfected with a plasmid containing the 1st exon of the *IT15* gene, carrying 74 CAG repeats (coding for a mutant huntingtin variant, mHTT) or 23 CAG repeats (encoding a healthy huntingtin variant, HTT). This model was used to perform preliminary tests, as it is currently the only cell model that allows to test genistein activity selectively on HTT and mHTT (because HD is an autosomal dominant disorder, there is a mixture of both huntingtin variants in cells derived from heterozygous patients). This approach is extremely important because mHTT leads to the disruption of many functions related to cellular processes, while healthy HTT plays important roles in the organism, as it has significant activities in vesicular transport, regulation of transcription, and prevention of apoptosis.

The results obtained during the implementation of a part of my doctoral dissertation related to HD showed that 48-h incubation of cells in the presence of genistein led to a definite reduction of aggregates of mHTT and its soluble form, and this decrease depended on the used concentration of this isoflavone. Similar tendencies were observed in the number and volume of mHTT aggregates. What is important and interesting, the level of the healthy form of this protein remained unchanged, which is a very positive feature in the light of the future use of the tested strategy in therapy. This also shows autophagy as a seemingly non-selective process which, however, is primarily responsible for the degradation of abnormal macromolecules, not removing wild-type proteins. In addition, studies using genistein indicated its positive effect on reduced proliferation and an increased number of apoptotic cells in cultures of HEK-293 transfected with plasmids encoding mHTT (compared to control cells that are transfected with a plasmid encoding a healthy HTT variant).

Obtained results were an introduction to the studies on the molecular mechanism of genistein-dependent degradation of mHTT. As mentioned earlier, the activation of TFEB by genistein, detected during studies on mucopolysaccharidosis, suggested that the autophagy process is the main mechanism of its action. My attention, therefore, was focused on examining the role of this process in lowering the level of mHTT. Immunodetection analysis of autophagy markers showed a significant increase in their level after incubation in the presence of genistein, what was indicative of the effective induction of autophagy in the tested cells. The increase in the autophagy marker level was accompanied by an increase in the number of lysosomes which also indirectly proves the stimulation of the studied process. The question was whether the drop in the level of mHTT and the induction of autophagy are two separate processes occurring side by side in cells, or one process is a consequence of the other. In order to investigate whether the autophagy process is responsible for lowering the level of mHTT under the influence of genistein, I used an inhibitor of autophagy - chloroquine, which works by suppressing the function of lysosomes. After treatment with chloroquine, the genistein-dependent degradation of mutant huntingtin turned out to be significantly less effective which indicates that autophagy is mostly responsible for the removal of pathogenic protein from the cells. The results of these studies, describing the potential of genistein in the treatment of HD and its mechanism of action, were published in the *Neuromolecular Medicine* journal [15].

To determine if the degradation of mHTT by genistein is not the result of a previously used model (in which exogenous mutant protein is delivered to cells), I also performed experiments on a model of cells taken from HD patients with a mixture of both HTT and mHTT. I carried out the experiments on 4 fibroblast lines collected from patients, characterized by the established disease as indicated by CAG repeats in the number of 40-43, and 4 lines of fibroblasts taken from healthy, age and sex-matched people (control cells). Microscopic visualization of HTT pointed to visible aggregates created by this protein in lines taken from HD patients, in contrast to control cells in which HTT did not form characteristic clusters of signals, generated as a result of antibody binding. Incubation of HD cells in the presence of genistein indicated a gradual disappearance of aggregates, which in the case of high concentrations of tested isoflavones were no longer noticeable.

The measurements of the HTT level in the tested cells turned out to be more complicated in the interpretation. Western-blotting analysis showed its increased level in cells collected from patients, whereas due to the lack of antibodies binding selectively to these two forms of the protein (HTT and mHTT), it is impossible to distinguish the variant of mutant and healthy HTT. It may be possible to separate them in the case of a higher number of CAG repeats in the 1st exon of the *IT15* gene, which is most probable in mouse models, where the number of these repetitions can reach 160 (thanks to which the mass of the mutant protein is higher than the mass of wild-type protein). However, in the case of human fibroblasts taken from patients, where it usually does not exceed 45 repetitions, this is unlikely. One could speculate that the reason of the increased levels of HTT in HD patients' cells is the consequence of formation of the insoluble form of this protein that can not be removed and therefore accumulates in the cell. Such a theory would be consistent with literature data on the insoluble structures taken by huntingtin as a consequence of the appearance of an elongated polyQ sequence. Moreover, it is worth mentioning that it is confirmed by the fact that accelerated lysosomal degradation in cells stimulated by genistein removes an excess HTT to its level in control cells (in which the level of HTT under the influence of genistein remains unchanged).

These results, published in *Metabolic Brain Disease* [16], not only confirmed the results of studies performed on the HEK-293 cell model transfected with plasmid DNA encoding two HTT variants, but also indicated the effectiveness of genistein on a more complicated model, but at the same time better suited to the clinical conditions. However, the interpretation of these results is much more difficult.

***Studies carried out on the Alzheimer's model***

The cause of AD is the accumulation of toxic forms of β-amyloid (βA), forming amyloid plaques, and hyperphosphorylated tau protein (P-tau), being the main component of neurofibrillary tangles, in CNS structures, mainly in the area of cerebral cortex, hippocampus and amygdala [17, 18]. The precursor of β-amyloid is the APP (amyloid precursor protein) protein, which undergoes proteolytic transformation into β-amyloid under the action of secretases. However, the specific cause of β-amyloid and P-tau accumulation in the CNS is unknown. Literature data provide information on detected mutations in the gene encoding the APP protein in the secretase cleavage sites or in genes encoding the secretases themselves. However, these data refer only to the familial form of AD that has a genetic basis. The reasons for the accumulation of these proteins in the sporadic (non-inherited) form remain unknown [17, 18]. The main symptoms of the disease include abnormal abstract thinking or damage to semantic memory (early stages) and language difficulties, impaired long-term memory and personality changes (late stages). Patients exclude themselves from social and family life. They gradually lose their vital functions, which leads to death most often within 7 years of diagnosis [17, 18, 19].

It should be noted that genistein studies in AD have already been carried out on different models. Although the authors of some works demonstrated interesting results, it should be taken into consideration that none of these reports even mention about autophagy as the mechanism of genistein action. Some of these works focused on apoptosis [20], anti-inflammatory activities [21] or antioxidant properties of this isoflavone [22]. The experiments planned by me focused on a completely different genistein activity. These studies were the first to identify autophagy as a mechanism of genistein in lowering the level of βA or P-tau (which has never been proposed before) and allowed to determine the effect of genistein administered at a dose that stimulates autophagy for treatment effectiveness in AD models (previously published reports have been used much lower doses).

Unfortunately, the availability of reliable cellular AD models is very limited. That is why I decided to carry out the research on the animal model of this disease. I worked on a streptozotocin (STZ)-induced rat model of AD. STZ is a derivative of glucosamine and a nitrosourea produced naturally by *Streptomyces achromogenes*. When administered into rats intraventricularly, it penetrates the cells with the GLUT-2 receptor, causing the formation of reactive oxygen species, neuroinflammation (the occurrence of these phenomena was also found in AD patients) and finally - accumulation of amyloid plaques composed of βA and P-tau neurofibrillary tangles. As a consequence, the cholinergic transmission is disturbed, which leads to neurodegeneration mainly of the hippocampal region resulting in a significant deficit of memory processes. This model is a well-documented, representative model of AD [23].

Wistar rats were divided into 4 groups:

1) VEH + WATER: with intraventricular injection of solvent for STZ, treated with water (n = 12);

2) VEH + GEN: with intraventricular injection of solvent for STZ, treated with genistein (n = 12);

3) STZ + WATER: with intraventricular STZ injection, treated with water (n = 12);

4) STZ + GEN: with intraventricular STZ injection, treated with genistein (n = 12).

After 2 weeks of handling, the rats were subjected to a surgery during which intraventricular injection of STZ/solvent was performed. After a 2-day reconvalescence period, supplementation with water (control groups) or genistein at a dose of 150 mg/kg/day (dose inducing the autophagy process, data taken from experiments on an animal model of mucopolysaccharidosis) was started.

*Behavioral studies*

One month after the STZ or solvent (control) injection procedure, behavioral tests were performed to assess the locomotor activity of rats, measure the severity of anxiety or, especially important for AD, measurement of long-term memory.

I measured locomotor activity of rats in the actometers by summing the number of their horizontal, vertical and ambulatory movements during the 2-hour period. The results of the measurement showed that animals after STZ injection are characterized by significant motor hyperactivity. In contrast, the administration of genistein to these animals for a month completely abolishes this disease phenotype, making these animals indistinguishable from control (healthy) animals. Anxiety measurement results (using the elevated plus maze test) showed that AD-rats treated with genistein exhibited natural anxiety behavior as opposed to untreated rats in which physiological fear of height and space is reduced. The most comprehensive and the most important part of behavioral research were the results of a memory measurement test, which I estimated using the Morris Water Maze. The results obtained during this test proved that memory in rats treated with genistein is identical to the memory of control (healthy) rats, whereas rats after STZ injection suffer from severe cognitive disorders.

*Biochemical studies*

The results obtained during biochemical tests performed on the tissue material collected *post mortem* from rats showed that in animals injected with STZ, APP, βA and P-tau levels is increased up to 4 times. However, in the group of AD animals treated with genistein, the level of all of the above mentioned proteins is reduced to levels of these proteins in control rats (Western blotting) or almost

undetectable (immunohistochemistry) in all examined brain structures, i.e. hippocampus, cortex and the rest of the brain. Similar results were obtained when detecting short sections of βA (βA40 and 42), which are currently considered to be the most toxic to nerve cells.

Turning to the mechanism of action of genistein, it should be emphasized that immunodetection of autophagy markers showed their elevated level in both groups of rats treated with genistein (both healthy and AD animals), which indicates an efficient induction of autophagy under the influence of this isoflavone. These groups also had an increased number of lysosomes.

The initial problem was to investigate whether the autophagy process is responsible for lowering the levels of APP and βA under the influence of genistein. In the case of animal models, it is not possible to inhibit certain processes (e.g. autophagy, what was done during research on a cellular HD model) because due to crucial role of this processes in organism, it can lead to death of an animal. Therefore, in order to check whether genistein causes degradation of APP and βA by induction of the autophagy process, I carried out experiments on a model of HEK-293 cells transfected with a plasmid containing the gene coding for the APP protein. It should be noted that there are no plasmids available that carry the gene encoding the mutant variant APP, so I used the plasmid with the wild type APP coding gene. Even if it was possible to carry out experiments with a plasmid with a gene encoding the mutated APP variant, it is worth remembering that the mutations in APP are the cause of only a part of cases with familial AD (which alone causes only 15% of AD cases, the rest is sporadic form of the disease). The other mutations concern the secretase-coding genes, and the use of this aspect and attempts to transfect plasmid DNA cells with a gene coding for mutant secretase variants would create an extremely complicated system that would lose its credibility at this point. The possible achievement of defective cells in the secretase-encoding genes would be possible using of the CRISP/Cas9 technique. Models of this type are very rarely used in research on AD yet and they would still be a model for testing only part of the cases of this disease.

HEK-293 cells transfected with the APP-encoding plasmids were incubated in the presence of genistein, and genistein and chloroquine over a 24 h period. The results showed a significant reduction in APP and βA levels under the influence of genistein compared to control cells, while inhibition of lysosomal function by chloroquine resulted in increased levels of these proteins suggested that in the AD model, as in the case of HD, autophagy is the mechanism responsible for the degradation of these toxic proteins under the influence of genistein. The results of the part of my dissertation focused on research on the rat model of AD are described in an article published in *Neuropharmacology* [24].

In conclusion, the results obtained during the course of my doctoral thesis indicated the great potential of genistein in the treatment of neurodegenerative diseases, due to its effectiveness in the degradation of pathogenic proteins that are the causes of these diseases as a result of the induction of autophagy. Considering the available literature data on genistein, and in particular the safety of its use in high doses and crossing the blood-brain barrier, it is suggetsed that genistein meets all the requirements for a therapeutic agent of this group of neurological disorders.

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