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INFLUENCE OF EXTRACT AND PHENOL GLYCOSIDES FROM ROSE PETALS ON THE FIBRILS OF AMYLOID PEPTIDE Aβ(1-42). STUDY BY TRANSMISSION ELECTRON MICROSCOPE

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The analyses of conformational diversity of Aβ(1-42) peptide aggregates by transmission electron microscope (TEM) visualization evidenced the simultaneous presence of non mature small protofibrils, mature fibrils and ribbon-like structural forms. In the presence of extract and phenol glycosides from rose petals, the deformations of aggregates and the amorphous bundles without clear structure were registered. The observations are in line with the ability of plant preparations to hinder the aggregation of Aβ(1-42) and to disaggregate the preformed aggregates. The work manifested that TEM is a reasonable approach in seeking effective agents for treatment of amyloid diseases and evaluating plant medicines as such agents.

***Keywords*:** amyloid beta 1-42 peptide, transmission electron microscopy, amyloidosis, plant preparations.

Introduction. The non-physiological aggregation and misfolding into insoluble amyloid fibrillar structure of proteins (beta-amyloid, alpha-synuclein, huntingtin and ataxin, superoxide-dismutase 1, tau and amylin), underlie more than 30 amyloid diseases and is responsible for several neurodegenerative pathologies (Alzheimer's, Parkinson's and Huntington's diseases, Amyotrophic Lateral Sclerosis, Frontotemporal Lobar Degeneration and Type 2 Diabetes). Alzheimer's disease (AD) is an incurable, degenerative fatal disease, characterized by progressive impairment in memory, cognition and behavioral functions. It is the most common form of dementia in the elderly. AD is associated with neuronal cell loss and synaptic dysfunction and is characterized by increasing accumulation of fibrillar tangles in neurons, amyloid fibers in neurotic plaques and in the walls of blood vessels. Aggregation and deposition of amyloid-β peptides (Aβ-peptides) and tau proteins are central events in the pathogenesis of AD [1].

The 42 residue amyloid β-peptide (Aβ42) is the predominant form found in plaques. While the monomeric Aβ42 is not neurotoxic, it forms toxic aggregates accumulating in the AD brain. Under physiological condition the ratio between Aβ42 and amyloid-beta (1-40) peptide (Aβ40) is about 1:10 [2]. However, Aβ42

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has a much greater neurotoxicity than Aβ40, and its aggregation kinetics is faster than other beta-peptides [3]. One of the reasons might be the increase of Aβ42 production relative to Aβ40 as a result of mutations owing to AD [4]. On a molecular length-scale, Aβ-peptides form aggregates of different shape under different *in vitro* conditions such as small, non-mature, diffuse oligomers and protofibrils, mature fibrils and their aggregates. All of these formations evoke neurological dysfunction [5]. The *in vivo* environment of Aβs is complex, and the question is: how the environmental parameters influence the aggregation process and the distribution of toxic and non-toxic Aβ conformations. Nonetheless, the analysis of dynamics of aggregation in simplified and controlled conditions *in vitro* can contribute to the knowledge on this process *in vivo*.

The inhibition of amyloid aggregation, increase of the decay and destruction of the formed aggregates are the main approaches in the AD treatment. The bioactive compounds, extracted from medical plants showing neuroprotective properties are of great interest in treatment of brain neurodegeneration [6]. Many *in vitro* researches indicated that polyphenolic compounds of plants may directly bind to Aβ42, interfering with its aggregation, changing oligomer conformation and attenuating cytotoxicity [7]. Particularly, the extract from rich with flavonoids *Rosa damascene* reversed behavioral deficit in a rat model of Aβ-peptides induced AD, manifesting a potential for prevention and treatment of cognitive dysfunction [8]. Kim and Oh [9] examined the researches on herbal medicines for AD, discussing the pros and cons of developing herbal anti-AD agents. They concluded that herbal medicines can be useful as AD-preventive agents, and "synergistic therapeutics, combining conventional medicine and herbal medicine for AD, may emerge in the not-so-distant future".

In our previous works the truncation of Aβ40 and Aβ42 peptides and slowing their aggregation/fibrillation in the presence of the enzyme dipeptidyl peptidase IV was demonstrated using transmission electron microscope (TEM) analysis [10]. The *in vitro* inhibition by plant preparations of aggregation of these peptides and disaggregation of their preformed aggregates was demonstrated using Thioflavin-T fluorescence technique [11]. The IC_{50} values in some of these processes were evaluated.

The present work studies the *in vitro* effects of ethanol extract from rose petals and isolated from it phenol glycoside fraction on Aβ42 fibrils using TEM technique.

Experimental Part. Thioflavin T (ThT) was purchased from "Sigma Ltd" (USA), G-25 and LH-20 Sephadex was purchased from "Pharmacia Biotech" (Uppsala, Sweden) and Aβ42 peptide was purchased from "China peptide" (CPR). All the other chemicals were of the highest purity. Spectral measurements were performed on spectrophotometer Specord M-40 UV-VIS (Germany) and spectro-fluorometer Perkin-Elmer MPF-44A (USA).

Electron Microscopy. On copper grids coated with formvar, the suspension of the preparation was applied drop-wise, in 1 *min* the liquid was removed and the specimen was stained with 1.0% phosphorous tungsten solution, pH 7.2. TEM images of the specimens were registered in the JEM-1400PLUS TUNGSTEN, operating at accelerating voltage of 80 *kV*, provided with the image recording digital system.

Plant Material. The rose petals *(Rosa damascena*) were purchased from Phytotherapeutic Center "Artemisia" (Armenia) and dried in the shade. The dried material was grinded, and 10% (w/v) extract was prepared in 70% (v/v) ethanol (72 *h* at ambient temperature). The extract was filtered through a sterile cheese cloth, dried by evaporation at 37°*C* and stored at –18°*C*. To obtain the fractions, 1–3 *mg* of the extract was dissolved in 2 *mL* of 70% ethanol and subjected to sequential gel filtrations on LH-20 and G-25 Sephadex columns. The constituents of the extract and the isolated fractions were characterized by optical absorbance in UV-Vis region, identified by qualitative chemical analysis and thin layer chromatography [12].

Peptide Preparation. Due to low solubility in water, 1 *mg* of Aβ42 was dissolved in 0.1 *M* NaOH, immediately neutralized with diluted HCl and 20 *mM* phosphate buffer, pH 7.2, and centrifuged (5000 $g\times10$ *min*) after standing for 30 *min*. The molar concentration of peptide was evaluated from the absorption spectrum, using the extinction coefficient of tyrosine at 276 *nm*, 1.39 $mM^{-1}cm^{-1}$.

Fibrillation/Aggregation of Aβ42. The fibrils of Aβ42 formed at incubating protein at concentration of 100 μM in 20 *mM* HEPES buffer, pH 7.2, containing 0.02% Na azide (w/v) for 7 days at 37°*C*. These conditions ensure the formation of aggregates, as it was demonstrated earlier by TEM analysis [10]. The fibrillation state of peptide was evaluated by ThT staining and measuring the fluorescence intensity at λ_{ex}=430 *nm* and λ_{em}=485 *nm* [13, 14]. Both the extraction and emission slits were studied at 6 *nm*.

Influence of Plant Preparations on Aβ42 Aggregation. To evaluate the effects of ethanol extract and phenol glycoside fraction of rose petals on the Aβ42 fibrillation, the identical solutions of peptide were incubated in the absence (control) and presence of these plant preparations at appropriate concentration. The fibrillation state of the experimental sample was expressed as its ThT-fluorescence percentage of the control fluorescence.

Statistical Analysis. The data, obtained at least in three independent experiments, were analyzed using the software InStat, version 3 for Windows (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was accepted for one-tailed p-value < 0.05.

Results. Fig. 1 shows the TEM image of a specimen of freshly prepared solution of Aβ42 in 20 *mM* HEPES buffer, pH 7.2. Here, only a few mature fibrils are seen (indicated by arrow 1). The diameter of fibrils is 52 *nm*.

The incubation of Aβ42 solution during 7 days in HEPES buffer pH 7.2 at 37°*C*, results in fibrillation-aggregation of peptide. Fig. 2 shows the TEM micrograph of this suspension. Many aggregations of fibrils (ribbon-like, indicated by arrow 1) and of non-mature protofibrils (indicated by arrows 2) are seen in this micrograph. On the micrograph in Fig. 1 such formations are not seen.

Figs. 3 and 4 present the TEM micrographs of Aβ42 samples after 7-day incubation in HEPES buffer, pH 7.2, at 37°*C* in the presence of 200 $\mu g/mL$ of ethanol extract from rose petals and of phenol glycoside fraction isolated from this extract respectively. The fibrils, indicated in micrographs 3 and 4 by arrows 1, are wider and more amorphous compared with the fibrils in Fig. 2 (in Fig. 4 more than in Fig. 3). Here, the number of fibril aggregations is diminished notably, and the non-mature protofibrils like of those in Figs. 1 or 2, are not seen.

The peculiarities of TEM images in Figs. 3 and 4 are in accordance with the lower aggregation of Aβ42 in the presence of phenol glycoside from rose petals, registered by ThT-fluorescence analysis. However, the high concentrations of plant preparations, used for preparing the TEM samples, did not allow using ThT-fluorescence analysis due to the interference of their optical absorbance.

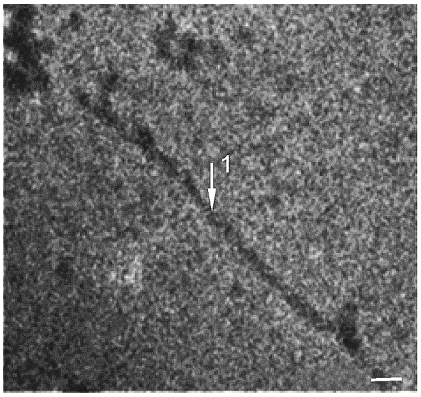

Fig. 1. The micrographs of TEM image of Aβ42 specimen as 100 μM solution in HEPES buffer, pH 7.2. Arr. 1 indicates a rear seen fibril. Scale bar: 100 *nm*.

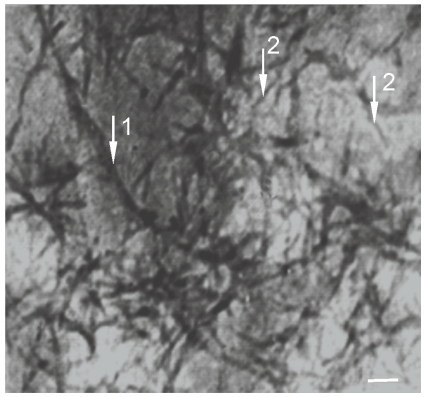

Fig. 2. The micrograph of TEM image of Aβ42 specimen after incubating during 7 days, 37°*C*, in HEPES buffer, pH 7.2. Arr. 1 indicates the ribbon-like aggregations of fibrils; arr. 2 indicate the non-mature protofibrils. Scale bar: 100 *nm*.

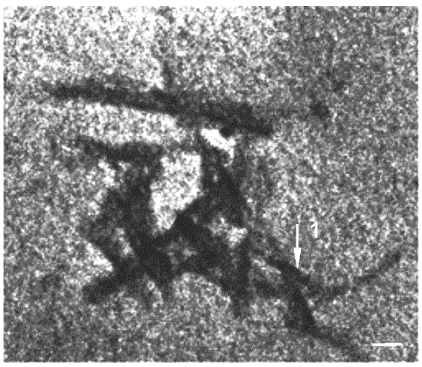

Fig. 3. The micrograph of TEM image of Aβ42 peptide after 7-day incubation in the presence of the ethanol extract from rose petals (200 $\mu g/mL$) in HEPES buffer, pH 7.2. Arr. 1 indicates the fibril aggregation. Scale bar: 100 *nm*.

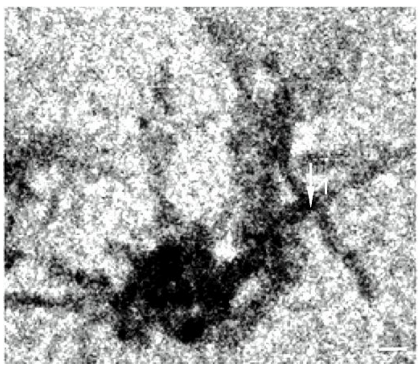

Fig. 4. The micrograph of TEM image of Aβ42 after 7-day incubation in the presence of phenol glycoside fraction from rose petals (200 $\mu g/mL$) in HEPES buffer, pH 7.2. Arr. 1 indicates the fibril aggregation. Scale bar: 100 *nm*.

In special experiments, three identical samples of Aβ42 were incubated in the absence (control) and in the presence of two low concentrations (2.5 and 7.5 $\mu g/mL$) of phenol glycoside fraction from rose petals. The ThT-fluorescence analysis showed that the plant preparation in this concentrations inhibited Aβ42 aggregation down to 40 and 50% of the control sample respectively ($p<0.05$).

Besides, the ability of phenol glycoside fraction to disaggregate the preformed Aβ42 aggregates was monitored using ThT-fluorescence analysis. Two identical suspensions of preliminary aggregated Aβ42 peptide were incubated for 3 days in

the absence (control) and presence of 7.5 $\mu g/mL$ phenol glycoside fraction from rose petals. ThT-fluorescence analysis registered diminishing of the aggregation state of the sample with plant preparation down to 70% of the control ($p<0.05$).

Discussion. The transmission electron microscopy analysis of conformational changes in Aβ42 suspension in the aggregation conditions manifested simultaneous presence of various structural forms: non-mature protofibrils, mature fibrils and fibrillar aggregations. All of these formations are toxic for neuronal cells [5].

The presence of ethanol extract and phenol glycoside fraction of rose petals in the incubation medium brought to the increase of the sizes of peptide fibrils, presumably, due to involving the components of the plant preparations, as we observed earlier in amylin fibrils [15]. For special note is the disappearance of small protofibrils and oligomers, which are known as more toxic formations of amyloid peptides, inducing apoptosis of neuronal cells [16].

The described observations can be considered as evidences of loosening of the fibril structure in the presence of the studied plant preparations. Hence, these preparations can appear as factors, promoting decomposition of fibrils. In our previous researches, the ability of several plant extracts (of rose petals among them) and of their fractions (phenol glycosides among them) to inhibit the self-aggregation of Aβ40 and Aβ42 amyloid peptides and to disaggregate their preformed aggregates was demonstrated [11]. Particularly, in disaggregating of Aβ40 aggregates, rather low IC_{50} values have been estimated for ethanol extract from rose petals (11.6±1.0 $\mu g/mL$) and the isolated from it phenol glycoside fraction (7.1±1.7 $\mu g/mL$).

We described similar properties of these plant preparations upon studying the *in vitro* action on a peptide hormone amylin, crucial for pancreatic islet β-cells. Several of the studied plant preparations inhibited the aggregation and promoted the disaggregation of preformed aggregates of this peptide, whose amyloidosis concern the development of type 2 diabetes mellitus [13, 14, 17]. These observations were proved by TEM and SEM analyses [15].

The results of this work assert: a) the ability of the studied plant preparations to prevent the toxic amyloidosis; b) the consideration of high resolution transmission electron microscopy is a reasoned approach in the search of anti-amiloidogenic agents.

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