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Fullerene C_{60} Prevents Neurotoxicity Induced by Intrahippocampal Microinjection of Amyloid-β Peptide

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The dynamics of the state of hippocampal pyramidal neurons after intrahippocampal microinjections of (1) amyloid-β_{25-35} (1.6 nmol/1 μl), (2) an aqueous molecule-colloidal solution of C_{60} (0.46 nmol/1 μl) and (3) an aqueous molecule-colloidal solution of C_{60} before amyloid-β_{25-35} administration were analysed in rats. This model allowed us to study the role of amyloid-β_{25-35} in the pathogenesis of Alzheimer's disease and to test anti-amyloid substances. Methods of fluorescent (acridine orange) and brightfield (cresyl violet and immunohistochemistry) microscopy were used. Acridine orange staining indicated changes in protein synthesis intensity due to alterations in the rRNA state of neuron ribosomes. One day after administration of amyloid-β_{25-35}, the intensity of protein synthesis in the population of morphologically intact cells decreased by 45%. By day 14, degeneration occurred in the majority of pyramidal cells, and amyloid-β_{25-35} deposits were observed in the neuronal cytoplasm. In necrotic cells, acridine orange staining of the cytoplasm was drastically increased as a result of RNA degradation rather than due to an increase in protein synthesis. Because amyloid-β_{25-35} administration provoked oxidative stress, we assumed that an aqueous molecule-colloidal solution of C_{60} administered before amyloid-β_{25-35} prevented protein synthesis changes on day 1, while acting as an antioxidant, and by day 14 it inhibited neurodegeneration and amyloid-β_{25-35} accumulation. Based on the data that an aqueous molecule-colloidal solution of C_{60} prevented amyloid-β_{25-35} aggregation in *in vitro* experiments and based on our present evidence on the antitoxicity of an aqueous molecule-colloidal solution of C_{60}, we suggest that functionalised C_{60} prevents/diminishes amyloid-β_{25-35} aggregation *in vivo* as well. Thus, an aqueous molecule-colloidal solution of C_{60} administered at a low concentration before amyloid-β_{25-35}, prevented disturbances in protein synthesis, neurodegeneration and formation amyloid-β_{25-35} deposits in hippocampal pyramidal neurons *in vivo*. This evidence gives promise that functionalised C_{60} can be used to develop anti-amyloid drugs combining antioxidant and anti-aggregative properties.

Keywords: Aqueous Molecule-Colloidal Solution of C_{60}, C_{60} Hydrated Fullerene, Anti-Amyloid Drugs, Alzheimer's Disease, Neurodegeneration, Protein Synthesis, Pyramidal Neurons, Hippocampal CA1 Field.

RESEARCH ARTICLE

1. INTRODUCTION

Alzheimer's disease (AD) is a primary neurodegenerative disorder in elderly individuals, and is characterised by progressive memory decline and dementia. An increase in the concentration of amyloid-β peptide (Aβ) 39–43 in brain neurons is a key factor in AD pathogenesis. The hippocampus and the frontal cortex are disturbed at early stages of the disease.[1–4]

The treatment of AD is a challenge of both psychiatry and neurology. No drugs exist to arrest the development or induce long-term remissions of AD. Recently, strategies aimed at the creation of anti-amyloid drugs, which, in particular, prevent aggregation of Aβ, have been developed.[4, 5] Fullerenes have not received attention in these studies.

C_{60} fullerenes are carbon nanoparticles with unique physicochemical and biological properties. One of the main biological properties of C_{60} is the ability to quench reactive oxygen species and to behave as a sponge for free radicals.[6–11]

Fullerenes are accumulated in liver, spleen and some other organs of experimental animals.[12] Recently, it has been shown that fullerene C_{60} can be eliminated.[13] Fullerene molecules in a low aggregation state, such as C_{60}HyFn and C_{60} polyvinylpyrrolidone, are nontoxic.[8, 14, 15] C_{60}HyFn remove hydroxyl radicals and protect

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DNA against oxidative damage induced by ionizing radiation *in vitro*.[11] The novel polyhydroxylated fullerene (C (60) (OH) (44) 8H (2) O: SHH-F) protect human keratinocytes from UV-induced injuries and decrease intracellular ROS generation and DNA damage.[16]

An interesting hypothesis about the mechanisms of fullerene action was suggested by Andryevsky and coauthors.[11] They proposed that special clustered water structures ordered by C_{60} fullerene molecules are the basis for effective deactivation of free radicals. However the mechanisms action of C_{60} still remain unclear.

Functionalised fullerenes have drawn great attention as a new class of antioxidant which also show neuroprotective activity *in vitro* and in animal models of Parkinson's disease, familial amyotrophic lateral sclerosis and local brain ischemia.[17–20] The international pharmaceutical companies C. Sixty and Merck Co. have started the development of antioxidants and drugs based on fullerenes to treat Alzheimer's disease and other neurodegenerative diseases.[21]

Fullerenes acted on a variety of cellular and molecular targets.[8, 22, 23] Carboxyfullerene prevented apoptosis induced by $A\beta_{1-42}$.[17] Fullerenol blocked $A\beta_{25-35}$-induced increases in cytosolic free calcium in cell cultures.[24] An *in vitro* fluorescent analysis showed that fullerene efficiently decreased the aggregation of $A\beta_{1-40}$. The authors explained this effect by binding of the central hydrophobic motif KLVFF of Aβ by fullerene.[25] Recently, using the method of high resolution electron microscopy, we have shown that several C_{60} fullerenes are able to prevent aggregation and to destroy $A\beta_{25-35}$ and $A\beta_{1-42}$ fibrils.[26, 27] These data have allowed us to suggest that Aβ peptides represent a target for fullerenes.[28] However, it is unknown as to whether fullerenes protect neurons against the toxic effects of Aβ *in vivo.*

Pyramidal cells of the hippocampal CA1 field are most sensitive to $A\beta_{25-35}$,[29] a neurotoxic fragment of $A\beta_{1-42}$.[30] After central administration of $A\beta_{25-35}$, accumulation of endogenous Aβ and degenerative alterations in CA1 field neurons has been shown to occur. It is believed that these alterations induced disturbances in cognitive processes.[29, 31, 32]

Aβ also induces the formation of reactive oxygen species and oxidative stress, an essential contributor to the early stages of AD pathogenesis.[33–35] The main targets of oxidative stress are the components of protein synthesis (messenger RNA, ribosomes, transfer RNA and chromatin).[36, 37]

In this work, we performed dynamic studies on the ribosomal RNA (rRNA) state in actively synthesising polyribosomes and monosomes in hippocampal CA1 pyramidal neurons after administration of $A\beta_{25-35}$ using the fluorescent dye acridine orange (AO). The ratio of its red and green fluorescence reflects the state of rRNA in the ribosomes and correlates with the share of total ribosomes represented in active polyribosomes, which allows for an estimation of protein synthesis intensity.[38, 39]

Since the toxic effect of Aβ is manifested at early stage of AD, it is important to investigate this stage in an animal model following intrahippocampal administration of Aβ. This model will be used to examine the feasibility of prevention of neurodegeneration and protein synthesis disturbances by means of functionalised fullerenes.

So, the objectives of the present work were to study
(1) neurodegeneration and the state of rRNA in the cytoplasm of rat hippocampal CA1 pyramidal neurons at different times (days 1, 7, 14) after intrahippocampal microinjection of $A\beta_{25-35}$ and
(2) the ability of C_{60}FWS to prevent these disorders.

2. METHODS

2.1. Chemicals and Characterization of C_{60} Hydrated Fullerene

C_{60} hydrated fullerene (C_{60}HyFn), a highly stable fullerene water solution (C_{60}FWS), was granted to us by Andrievsky and co-authors, the developers of this substance. C_{60}HyFn has been synthesized by using a special technology for transfer of fullerene molecules (MER Corp., USA, purity N99.5%) from their solution in an organic solvent into an aqueous phase by sonication without addition of any solubilizers and stabilizers.[11, 40] The concentration of C_{60}HyFn was 460 μM (0, 3 mg/ml). C_{60}HyFn solutions, depending on their concentration, contain both single C_{60}HyFn molecules ($C_{60}@\{H_2O\}n$ or $C60@nH_2O$, where $n = 22–24$) and their labile clusters of 3–72 nm. C_{60}HyFn combines the properties of both colloidal systems and solutions of weak polyacids and/or their inorganic salts with the highest buffer capacity near the physiological pH value. It holds stable without aggregation for several years.

$A\beta_{25-35}$ (Sigma, USA) was diluted in distilled water and aggregated for 24 h at 37 °C. The aggregation degree of Aβ was determined by electron microscopy.

Administration of 0.9% NaCl solution served as a control.

2.2. Tissue Sample Preparation

Cytological investigations were performed on five animal groups at days 1, 7 and 14 after drug administration:
(1) intact control, $n = 3$, where n is the number of animals;
(2) injection of 0.9% NaCl solution, $n = 3$;
(3) injection of $A\beta_{25-35}$, $n = 3$;
(4) injection of C_{60}HyFn, $n = 3$;
(5) injection of C_{60}HyFn and then $A\beta_{25-35}$, $n = 3$.

After decapitation, a part of brain was fixed in Carnow mixture (ethanol, chloroform, acetic acid, 6:3:1,

RESEARCH ARTICLE

respectively) and the other part in 4% parafolmaldehyde in PBS and embedded in paraffin. Seven micron-thick sections were cut from blocks using a microtome (Leica, Germany). Sections were used for fluorescent (AO) and brightfield (cresyl violet and immunohistochemistry) microscopy.

2.3. Fluorescent Microscopy

To study the state of rRNA in the neuronal cytoplasm, brain sections were stained with AO (Fluka Chemia AG, Buchs, Switzerland) under conditions optimally suited to the dye-RNA interaction. After dewaxing and rehydration, sections were rinsed with citrate-phosphate buffer, pH 4.2 for 4 minutes and stained with AO at a concentration of 0.3 mM in citrate-phosphate buffer, pH 4.2, for 10 min according to a technique described earlier.[38] AO monomers form complexes with double-stranded RNA regions, which fluoresce at 530 nm (I_{530}), whereas AO dimers bind to single-stranded RNA regions and fluoresce at 640 nm (I_{640}). The measurements were carried out on a DMF-2 microfluorimeter (Pushchino, Russia).[41] Fluorescence in the cytoplasm only was measured by recording probes 6.5 μm in diameter with a high-power 85× objective. Owing to the large volume of the cytoplasm, pyramidal neurons of the hippocampus are well suited for this purpose. Measurements and data processing were performed using the Microfluor software program (Pushchino, Russia). At each point, 300 cells were examined.

To analyse the cytoplasm, the coefficient $K_\alpha = (I_{640})/(I_{530})$ was used which represents the ratio of red and green fluorescence. Earlier, it was shown that K_α characterises the state of rRNA in ribosomes and correlates with the share of actively functioning ribosomes (polyribosomes), which allows for an estimation of protein synthesis intensity.[38]

2.4. Brightfield Microscopy

Degeneration of neurons was revealed by staining of sections with cresyl violet (Sigma-Aldrich, St. Louis, USA) (Nissl staining).

The immunohistochemical study was performed according to a standard technique.[31] To increase detection of Aβ, slices were treated with 70% formic acid for four

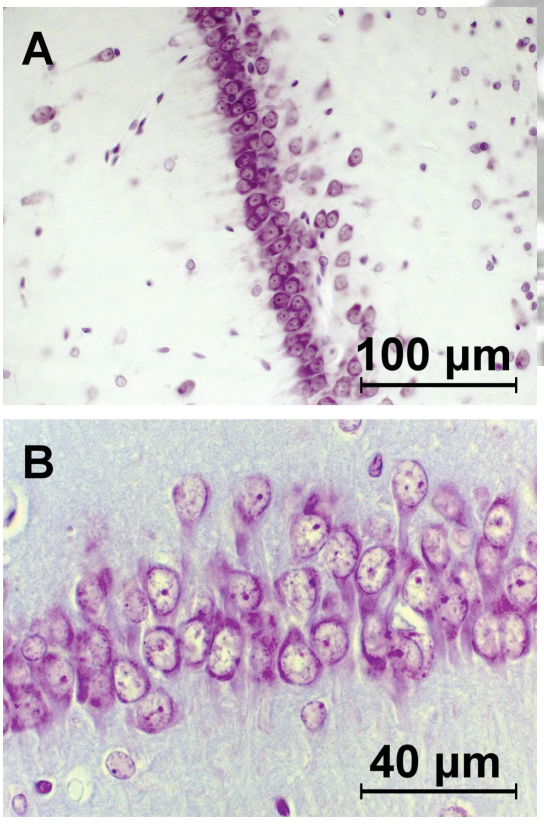

Fig. 1. Photomicrographs of the hippocampus (CA1 field) stained with cresyl violet in the control (intact rat).

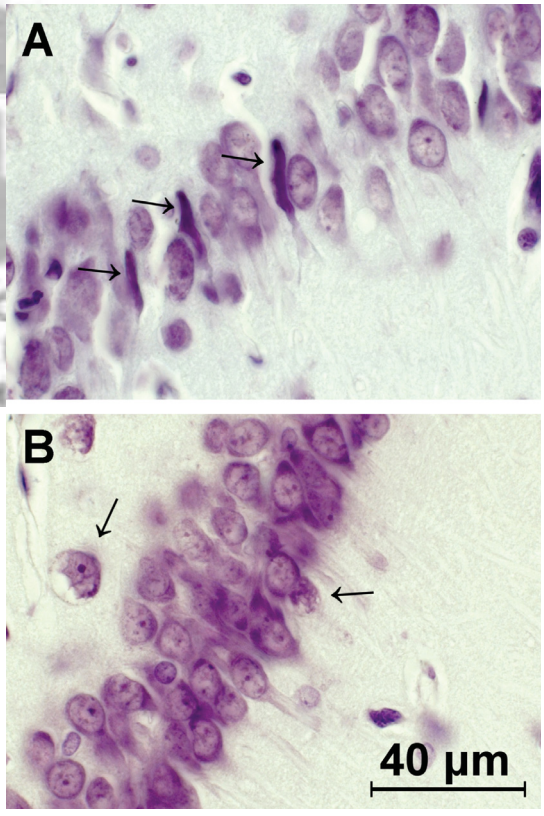

Fig. 2. Photomicrographs of rat hippocampus (CA1 field) stained with cresyl violet after injection. (A), (B) Aβ_{25-35} (1.6 nmol/1 μl). Arrows point to dark and degenerating cells. Decapitation was performed 1 day after drug injection.

minutes. To block the endogenous peroxidase activity, the slices were incubated in 0.5% H_2O_2 in ethanol, and 5% normal goat serum was used to block unspecific binding. Then, the slices were incubated with the primary antibody rabbit polyclonal anti-Aβ_{1-40} (1:400, Sigma, USA) at a temperature of +4 °C for 15 hours. The primary antibodies were recognised by anti-rabbit IgG (1:800, Sigma, USA) which were identified with StreptABComplex/HRP (1:800, Sigma, USA). The immunohistochemical reaction was visualised with 3,3′-diaminobenzidine (Sigma, USA). As a negative control, primary antibodies were replaced with buffer.

Histological preparations were analysed by means of an Axio Imager M1 optical microscope (Zeiss, Germany).

2.5. Animals

The study was performed on 53 Wistar male rats (3 months old) under standard conditions with food and water *ad libitum* and under a natural light regime in the vivarium of the Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences. The experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.6. Stereotaxic Surgery

The surgery was performed in a stereotaxic frame under sterile conditions and with combined intramuscular narcosis (zoletil, 12 mg/kg and xylazine, 20 mg/kg). The chemicals were injected once bilaterally at the following coordinates: hippocampal CA1 field, AP = −4 mm; L = 3 mm; H = 3 mm[42] by means of a Hamilton microsyringe (Hamilton, Germany, 5 μl) at the following concentrations: Aβ_{25-35} 1.6 nmol/1 μl, C_{60}HyFn 0.46 nmol/1 μl, 0.9% NaCl solution was injected in a volume of 1 μl. In the group with combined drug administration, C_{60}HyFn was injected two hours prior to Aβ_{25-35} administration at the same concentrations as in the previous cases. The hippocampus was sectioned at 40 μm and stained with cresyl violet to examine the injection sites.

2.7. Statistical Analysis

The data presented are the mean ± SD. The data were analysed using Student's t-test; $p < 0.001$ was taken as indicative of statistical significance.

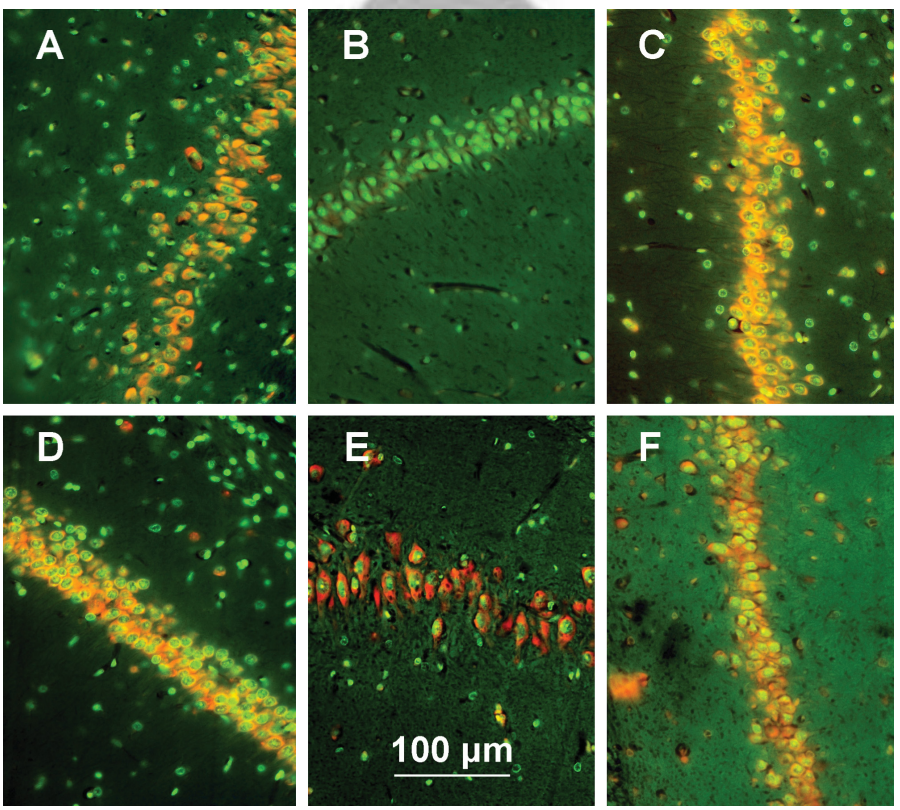

Fig. 3. Fluorescence of the cytoplasm in CA1 pyramidal neurons after intrahippocampal injection: (A) control (intact animals); (B), (E) Aβ_{25-35} (1.6 nmol/μl); (C), (F) C_{60}HyFn (0.46 nmol/μl) prior to Aβ_{25-35} administration; (D) C_{60}HyFn (0.46 nmol/μl); (B), (C) denote day 1; (D)–(F) denote day 14. The cytoplasm in pyramidal cells fluoresces in the red region, which reflects AO binding to single-stranded sites in rRNA.

3. RESULTS

The bulk of hippocampal CA1 field neurons are pyramidal cells.[43] Therefore we have limited our study to pyramidal cells. In the normal brain, these neurons, when stained with cresyl violet, show a uniformly stained cytoplasm and nucleoplasm, distinct plasmatic and nuclear membranes, and a well-defined nucleolus (Figs. 1(A, B)).

Cytological studies of CA1 hippocampal pyramidal neurons were carried out at 1, 7 and 14 days after intrahippocampal administration of (1) 0.9% NaCl solution, (2) $A\beta_{25-35}$, (3) C_{60}HyFn, and (4) C_{60}HyFn injected before $A\beta_{25-35}$.

Because the microinjections were accompanied by local damage of the brain, we separated this influence from the effect of the administered compounds.

One day after the injections, only a few dark and degenerating cells were observed in all groups in the study (Figs. 2(A, B)).

An investigation of hippocampal neurons stained with AO showed that 1 day after $A\beta_{25-35}$ administration, the red fluorescence of the cytoplasm decreased (Fig. 3(B)) compared to the control (Fig. 3(A)). In all CA1 field neurons studied, a significant drop in K_α, (55% of control) was observed, $p < 0.001$. The histograms for distribution of K_α values show a substantial increase in the number of cells with low K_α. In the case of C_{60}HyFn injected alone or before $A\beta_{25-35}$, all parameters studied remained at the control level (Figs. 3(C), 4.1.(C, D), and Table I).

Seven days after $A\beta_{25-35}$ administration, multiple shrunken cells and thinning of the cell layer in the region of the CA1 field adjacent to the site of injection were observed. Substantial number of cells showed signs of degeneration (Figs. 5(B–D)). In the case of C_{60}HyFn injected before $A\beta_{25-35}$, damaged cells were observed mainly at the injection site (Fig. 5(F)).

Hippocampal neurons stained with AO showed a significant decrease in K_α (77% of control) 7 days after $A\beta_{25-35}$ injection, $p < 0.001$. However, substantial differences between the histograms for 1 and 7 days after drug administration were noted (Figs. 4.2.(B) and Table I). In the case of injection of C_{60}HyFn alone and C_{60}HyFn prior to beta-amyloid, the K_α values did not differ from the control (Figs. 4.2.(C, D) and Table I).

Fourteen days after injection of $A\beta_{25-35}$ to the hippocampus, we have observed morphological signs of necrosis: significant swelling of the cytoplasm, vacuolisation, numerous indistinct and dark cells, as well as fully destroyed cells over the whole length of the CA1 field (Figs. 6(A–C)). A similar picture of neuronal degeneration in the CA1 field has been observed in hypoxia and ischemia.[43–45] After administration of C_{60}HyFn added before $A\beta_{25-35}$, there was no pronounced degeneration in the majority of CA1 field pyramidal cells (Figs. 6(D–F)). After injection with 0.9% NaCl or C_{60}HyFn, there was no distinction from the control (data not given).

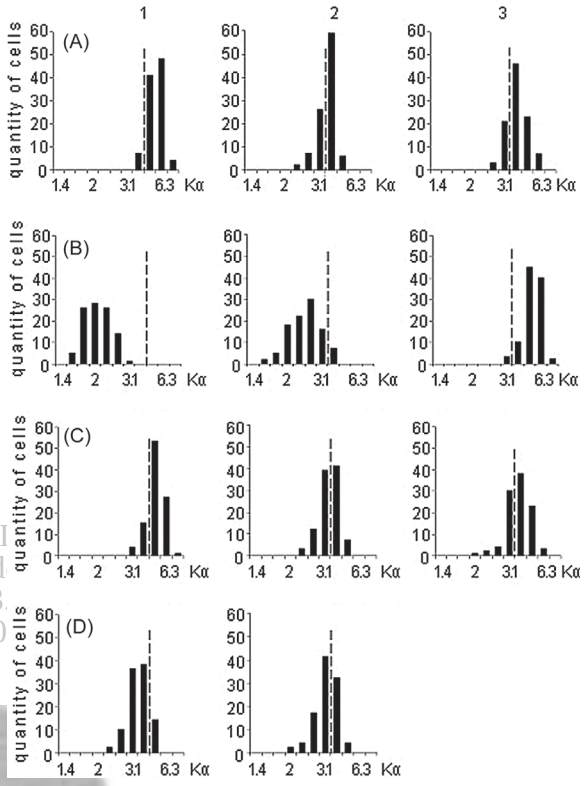

Fig. 4. Histograms of the value K_α for hippocampal CA1 field pyramidal neurons after intrahippocampal administration: (A) control (intact animals); (B) $A\beta_{25-35}$ (1.6 nmol/μl); (C) C_{60}HyFn (0.46 nmol/μl) prior to $A\beta_{25-35}$ administration; (D) C_{60}HyFn (0.46 nmol/μl). 1, 2, 3 denote 1, 7, 14 days after microinjections, respectively.

The investigation of hippocampal neurons stained with AO showed that 14 days after $A\beta_{25-35}$ administration, the red fluorescence of the cytoplasm was more intensive compared to the control (Fig. 3(E)). The value of K_α amounted 148% of control, $p < 0.001$. The histogram showed a substantial increase in the number of cells with high K_α (Fig. 4.3(B)). In the case of C_{60}HyFn injected alone or

Table I. Relative K_α^{exp} / K_α^{contr} values $\pm$ standard deviation (SD) after $A\beta_{25-35}$ injection and\or C_{60}HyFn compared to the control.

Time after administration	State of animal	K_α^{exp} / $K_\alpha^{contr} \pm$ SD ($n = 300$, where n is cell number)
Day 1	$A\beta_{25-35}$	55 ± 3^a
	C_{60}HyFn + $A\beta_{25-35}$	96 ± 3
	C_{60}HyFn	95 ± 3
Day 7	$A\beta_{25-35}$	77 ± 3^a
	C_{60}HyFn + $A\beta_{25-35}$	100 ± 3
	C_{60}HyFn	95 ± 3
Day 14	$A\beta_{25-35}$	148 ± 4^a
	C_{60}HyFn + $A\beta_{25-35}$	98 ± 4

[a]The statistical significance of difference between $A\beta_{25-35}$ and C_{60}HyFn + $A\beta_{25-35}$, $A\beta_{25-35}$ and intact control, $p < 0.001$.

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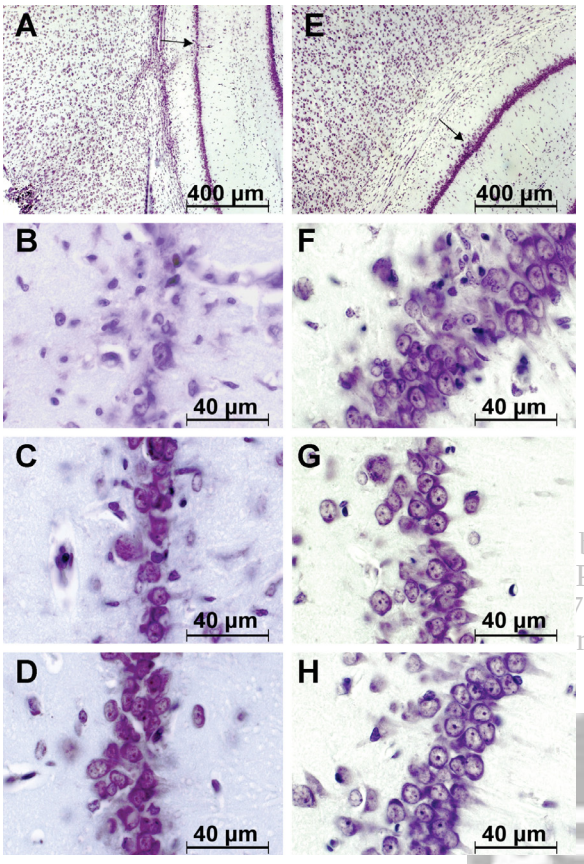

Fig. 5. Photomicrographs of rat hippocampus (CA1 field) stained with cresyl violet after injection of: (A)–(D) Aβ_{25-35} (1.6 nmol/μl); (E)–(H) C_{60}HyFn (0.46 nmol/μl) with subsequent administration of Aβ_{25-35}. Arrows show the mark of the injection needle. Decapitation was performed 7 days after drug administration.

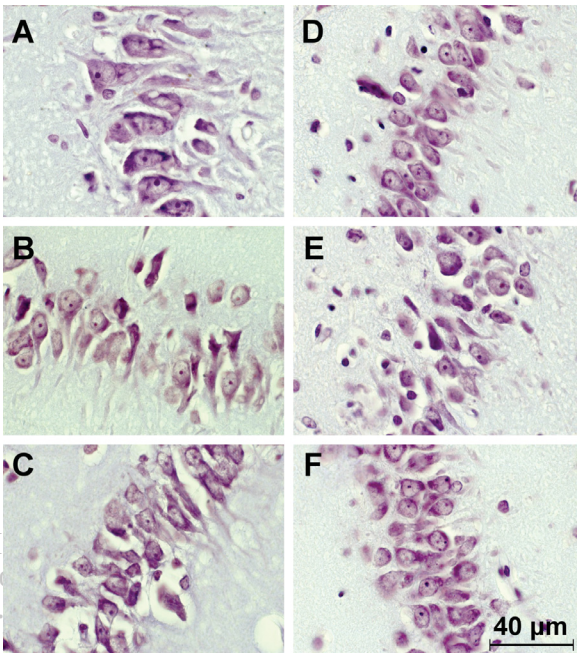

Fig. 6. Photomicrographs of rat CA1 field stained with cresyl violet after injection to the hippocampus of: (A)–(C) Aβ_{25-35} (1.6 nmol/μl), and (D)–(F) C_{60}HyFn (0.46 nmol/μl) prior to Aβ_{25-35}. Decapitation was performed 14 days after drug administration.

before Aβ_{25-35} (Figs. 3(F), 4.3.(C)), all parameters studied remained at the control level (Table I). The essential increase in K_α in the bulk of necrotic neurons of the CA1 field 14 days after Aβ_{25-35} injection as a result of RNA degradation, rather than protein synthesis increase.

Fourteen days after Aβ administration, an immunohistochemical study revealed the presence of diffuse Aβ deposits in the cytoplasm of CA1 field pyramidal neurons (Fig. 7(B)), whereas after days 1 and 7, no beta-amyloid deposits were seen (data not shown). In the case of C_{60}HyFn injected before Aβ_{25-35}, immunostaining occurred in only a few cells (Fig. 7(C)). In the control, staining of cells was not observed (Fig. 7(A)).

Thus, injection of at a low concentration C_{60}HyFn (0.46 nmol/1 μl) prior to Aβ_{25-35} injection prevented or reduced the development of neurodegenerative processes and changes in protein-synthesising activity, and moreover interfered with deposition of Aβ in the bulk of pyramidal cells.

4. DISCUSSION

Cytological analysis of CA1 field at early time points after intrahippocampal Aβ_{25-35} injection showed different degrees of pyramidal neuron degeneration at days 1, 7, and 14. Although degeneration was observed at day 1 only in neurons located at the site of the injection, the considerable decrease in K_α in all cells under study indicated an inhibition of protein synthesis.

Previously, we have shown that the value K_α correlates with the fraction of translating ribosomes and the decrease of this value does not depend on whether polyribosomal dissociation occurs or whether heavy ribosomes are formed.[38, 39] This fact is in agreement with the literature data where is has been shown that Aβ induces oxidative stress, leading to substantial decreases in protein synthesis in morphologically intact neurons due to the formation of heavy ribosomes.[36, 37]

Protein synthesis is the cellular processes most vulnerable to oxidative damage in AD.[36, 37] In *in vitro* experiments, it has been shown that oxidative stress induces increased expression of β- and γ-secretases, thus enhancing Aβ formation.[46–48] Fourteen days after Aβ injection, Aβ deposition in the cytoplasm of most CA1 pyramidal cells was accompanied by neurodegeneration (Figs. 6(A–C), 7(B)). An increased intensity of AO staining in cells suggests the degradation of ribosomes in these neurons (Fig. 3(E)). Intrahippocampal administration of C_{60}HyFn before Aβ

RESEARCH ARTICLE

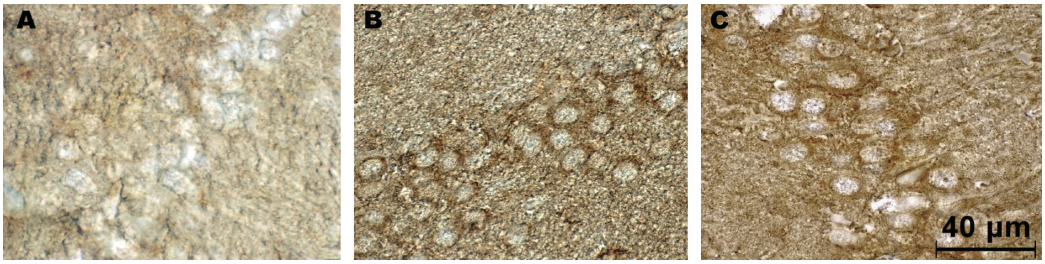

Fig. 7. Photomicrographs of rat hippocampal CA1 neurons. Amyloid deposits were identified by antibodies against $A\beta_{1-40}$: (A) control; (B) after injection of $A\beta_{25-35}$ (1.6 nmol/μl) to the hippocampus; (C) after injection of C_{60}HyFn (0.46 nmol/μl) prior to $A\beta_{25-35}$ (1.6 nmol/μl) administration.

prevented any degenerative alterations in neurons and inhibited the accumulation of Aβ deposits as well.

Also, substantial destructive changes in CA1 pyramidal neurons were observed in surviving hippocampal slices incubated in Aβ-containing media. C_{60}HyFn added to the medium prior to Aβ reduced the level of neurogeneration.[49]

At earlier stages of AD development and *in vitro*, oxidative stress significantly contributes to neuronal damage.[35–37, 50] Based on these data, we suggest that the protein synthesis decrease observed in our study in the first days after Aβ injection is caused by oxidative stress. The absence of changes in protein synthesis in the case of C_{60}HyFn administered before Aβ supports its antioxidant activity.

Aβ provokes the development of oxidative stress at early stages of AD. At later stages accumulation of Aβ deposits leads to severe neurodegeneration.[50] The absence of any substantial neurodegeneration and the less-pronounced accumulation of Aβ deposits observed in the case of preliminary injection of C_{60}HyFn may be associated with its ability to prevent the formation of Aβ fibrils and to destroy them. Earlier, we demonstrated this capability of C_{60}HyFn by *in vitro* experiments.[26, 27, 49]

Thus, we have found that C_{60}HyFn at low concentrations administered before $A\beta_{25-35}$ prevented the disturbance of protein synthesis, neurodegeneration and formation of Aβ deposits in hippocampal pyramidal neurons *in vivo*. Although the mechanism of the anti-amyloid action of C_{60} is the subject of further investigation, new evidence gives promise that functionalised C_{60} can be used to develop anti-amyloid drugs combining antioxidant and anti-aggregative properties.

ABBREVIATIONS

AD—Alzheimer's disease; AO—acridine orange; Aβ—amyloid-β peptide; C_{60}FWS—C_{60}fullerene water soluble; C_{60}HyFn—C_{60}hydrated fullerene; rRNA—ribosomal RNA.

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