

ANTI-AMYLOIDOGENIC EFFECT OF MiR-101 IN EXPERIMENTAL ALZHEIMER'S DISEASE

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The aim of the study was to determine the effect of miR-101 on the level of β -amyloid peptide and activation of the cytokine system in the brain regions of animals with an experimental model of Alzheimer's disease. MiR-101 is the key deactivating operator of mRNA function for the amyloid- β protein precursor. Hence, miR-101 is capable to suppress its synthesis and amyloidogenic processing. Aged male rats were injected intrahippocampally with single-dose unilaterally of β -amyloid peptide 40 aggregates (15 nmol). After 10 days, nasal administration of the liposomal form of miR-101 or empty liposomes was started. After 10 days of therapy, the level of toxic endogenous form β -amyloid peptide 42 and the activity of the cytokine system were determined by the indicators of tumor necrosis factor α , interleukin-6, and interleukin-10 in neocortex, hippocampus and olfactory bulbs. It was found that in rats, aggregates of exogenous β -amyloid peptide 40 model the amyloidogenic and pro-inflammatory situation after 20 days in the neocortex and hippocampus (a significant increase in the concentrations of β -amyloid peptide 42 by 36% and cytokines by 16–18% in the neocortex, and β -amyloid peptide 42 by 27%, proinflammatory cytokines tumor necrosis factor α , interleukin-6 by 14% in the hippocampus), but not in olfactory bulbs. The ten-day course of nasal therapy of liposomal miR-101 normalized the level of β -amyloid peptide 42 and cytokines: in neocortex, the concentration of endogenous toxic β -amyloid peptide 42 decreased by 33%, in the hippocampus by 15%, and concentration of pro-inflammatory cytokines fell by 11–20%. Thus, nasal therapy of miR-101 in liposomes caused a significant anti-amyloidogenic effect in rats with the Alzheimer's disease model, whereas its anti-inflammatory effect was primarily due to a decrease in β -amyloid peptide 42 concentration.

Key words: miR-101, β -amyloid peptide, amyloidosis, Alzheimer's disease.

RNA interference technologies are increasingly used for RNA-based medical preparations study. The reason for that is the possibility of controlling the expression of certain gene during the protein translation [1–3].

Alzheimer's disease (AD) is a type of dementia induced by amyloidosis [4–6]. The rare familial forms of AD with early onset are thought to be caused by the increased proteolytic production of β -amyloid peptide 42 (A β 42) from the amyloid precursor protein (A β PP). Pathogenesis of the common form of AD with late manifestation is still unclear. However, the enzyme BACE1, which is linked to amyloidogenic processing of A β PP, is more active in such patients [7]. That means that increased production and age-related

aggregation of A β 42 can promote a sporadic disease.

In [8–11], it was shown that mutations or gene polymorphism also regulate the A β metabolism in AD. By now, mutations of the following genes are linked to the early onset of AD: A β PP encodes the amyloid precursor protein, *PSEN1* encodes presenilin 1 (PS1) and *PSEN2*, presenilin 2 (PS2). Late onset of AD is associated with polymorphism of gene *AD2*, which encodes apolipoprotein E [12–13]. Several genes are also known to indirectly affect the amyloidosis regulation and are linked to that pathology. Those genes encode the low-density lipoprotein receptor (LRP), α -2-macroglobulin (α -2-M), insulin-degrading enzyme (IDE), ATP-binding

cassette transporter (ABCA1), cholesterol 24-hydroxylase (*cyp46*), etc. [14].

Most of the mutant genes in AD patients encode multifunctional proteins active in many branched biochemical pathways. This presents some difficulties for the pharmacological therapy aimed to correct the expression of such genes. Thus, using specific miRNA (also known as miR) to regulate the expression of target genes is a promising direction of research [15, 16].

MiRs are small (18–25 nucleotides), evolutionally conservative, non-coding, single-stranded RNAs which are key in various biological processes through regulating expression of target genes by binding with 3'-non-translated loci of their mRNA [17, 18]. Each miR is proven to control up to several hundreds of genes, and one gene can be a target for more than one miR [19]. These regulatory RNAs can “silence” a gene through various ways. First, they inhibit the gene expression by interacting with mRNA: miR attach to mRNA and block the translation process. Another way to deactivate a gene is during transcription, when miR, as part of poliprotein complex, induces epigenetic modifications in the genome: methylation of DNA and histones, and deacetylation of histones. Protein synthesis can also be inhibited by the interaction of miR with repressor proteins that block translation [20]. However, in very rare circumstances (namely, arrested cell cycle), miR, conversely, activates translation [21]. Hence, miRs are more and more used in diagnostics and therapy of neurodegenerative, cardiovascular, cancer and other pathologies [22].

MiR-101 belong to the family of miRNAs, which participate in several cellular activities such as cell proliferation, differentiation, invasion, and angiogenesis [23]. Hypoxia-sensitive miR-101 stimulates angiogenesis and factors in regulation of the vascular remodeling [24]. Deregulation by miR-101 is observed during the development of malignant neoplasms, which indicates its suppressor function in a number of tumor varieties [25]. MiR-101 regulates several simultaneous postnatal programs of brain development, because the balanced excitement/ deceleration is necessary for the normal functioning of neural networks [26]. Transitory loss of miR-101 regulation on pyramidal neurons in dorsal hippocampus causes the hypersensitivity of the neural network and cognitive deficit, thus concentration of miR-101 in the postnatal period is critical for further functioning of neural chains. This

miR inhibits NKCC1 chloride importer (gene *Slc12a2*) needed for initiating the maturing of GABA-ergic signaling system. That causes the reduced spontaneous synchronized activity and prevents dendrite overgrowth. Also, miR-101 is a part of program of development which activates the repression of motor protein 1A KIF1A (gene *Kif1a*) from the superfamily of kinesin and Ankyrin-2 (gene *Ank2*), to inhibit the excessive collection of pre-synaptic components and the decrease in the density of glutamatergical synapses. Targets of miR-101 also include mRNAs of the following genes: *Abca1*, *Ndr2*, *Slc7a11*, *PMCA2*, *Rapgef1*, *Slc25a4*, *Camk2a*, *Clasp2*, *Dbs*, etc. [26]. It is shown that miR-101 is a key operator of mRNA's function for A β PP (mRNA^{A β PP}) by deactivating it and inhibiting the protein synthesis of the amyloid- β protein precursor and its amyloidogenic processing [27–28]. In [29–31], the cytokine activation is shown to directly affect the expression of gene A β PP and A β PP synthesis during chronic inflammation in central neural system (CNS), accompanying the process of amyloidosis.

In previous studies of experimental AD rat model, it was shown that a natural polyphenol curcumin in soluble and liposomal forms inhibited the cytokine response to the toxic action of β -amyloid aggregates in target departments of animal brain (neocortex and hippocampus) [32–33]. A possible biochemical mechanism for this is that curcumin suppresses the activation of I κ B kinase (IKK), phosphorylation and degradation of I κ B α (inhibitor of NF κ B) and thus blocks the activation of nuclear transcription factor NF κ B [34–35]. The anti-inflammatory effect of curcumin causes improvement of mnemonic abilities and memory characteristics in animals with experimental AD. However, there is no evidence of direct and targeting inhibition effect of curcumin on excessive production of β -amyloid peptides.

The present work aimed to study the effect of liposomal miR-101 on the levels of β -amyloid peptide, and on the activation of cytokine system in brains of animals with experimental AD.

Materials and Methods

AD was modeled in aged male rats (14 months old) with intrahippocampal injections of aggregated Humanbeta Amyloid 1–40 protein (ChinaPeptidesCo., Ltd, China), as described previously in detail [26]. Commercial A β 40 was dissolved in bidistillate to the final

concentration of 15 $\mu\text{mol/l}$, and incubated at 37 °C for 24 hr for aggregation. Large A β 40 conglomerates were dispersed with ultrasound and sterilized directly before the injection. The suspension's volume was 10 μl per animal, infusion was carried out for 5 min. Stereotaxic coordinates of the area of injection in left hippocampus were determined using brain map in [40]. It corresponds to the distance from the intersection point of sagittal suture with bregma (zero point): 2 mm distally, 2 mm laterally and 3.5 mm in depth. Stereotaxic operations were performed on animals under general anesthesia with thiopental intraperitoneally (50 mg/kg). Intact animals served as control ($n = 6$).

Experimental AD rat model is generally accepted because it demonstrates not only the toxicity of A β aggregates (main mechanism of amyloidosis), but also the dementia symptoms characteristic for AD, such as worsening memory and violated mnemonic abilities [36, 37]. A β 40 was used instead of A β 42 to model AD in rats because even though the latter β -amyloid peptide is thought to be a specific marker of amyloidosis, A β 40 is synthesized in CNS by an order of magnitude more than A β 42 [39]. Thus, A β 40 aggregates are toxic for neural synapses. Also, A β 40 and A β 42 of rats do not aggregate, thus only A β 40 Human aggregates were used in experimental AD models.

In 10 days after the model was established, miR-101-3p (OOO "NPF Sintol", Russia) was nasally administrated to experimental animals ($n = 7$) and empty liposomes were given to rats from the comparison group ($n = 6$). Liposomes were obtained from lipid films [41]. In total, 10 therapeutic sessions were concluded; in each an experimental animal was given $2.5 \cdot 10^{14}$ molecules of miR-101, in single 20 μl doses of liposome suspension. Another group of rats with AD model ($n = 6$) were not given anything.

In 10 days of nasal therapy (20th day of experiment), all animals were decapitated. Neocortex, hippocampus and olfactory bulbs were removed in cold conditions, frozen and stored at -40 °C. Tissues of studied brain regions were homogenized in Tris buffer (50 mM tris-HCl, 150 mM NaCl, pH 7.5) and centrifuged at 14000 rpm for 5 min. Then, supernatant was collected. Supernatant samples of the aforementioned rat brain regions were used to determine concentrations of toxic endogenous form of A β 42, tumor necrosis factor α (TNF α), interleukin-6 (IL-6), and interleukin-10 (IL-10) in bioassay according to protocols of Rat Amyloid beta peptide 1-42 ELISA Kit (Bioassay Technology

Laboratory, China) for β -amyloid peptide 42, and Rat ELISA Kits TNF α , IL-6 and IL-10 (Invitrogen BCM DIAGNOSTICS, USA) for cytokines. Concentrations were expressed in ng/mg of protein for A β 42 and in pg/mg for cytokines. Absorption of samples was evaluated in microwell plate reader GBG Stat FAX 2100 (USA) at $\lambda = 450$ nm with wavelength correction at $\lambda = 630$ nm. Total protein content was measured according to Lowry [42].

Experimental protocols for rats were conducted in compliance with "General ethical principles of experiments on animals" (Kyiv, 2011).

The obtained results were statistically processed, average values and standard deviations were calculated. Statistical analysis of differences was done with Student's t-test for samples with normal distribution. Values were considered significant at $P < 0.05$.

Results and Discussion

1. Anti-amyloidogenic effect of miR-101

It was shown that the introduction of A β 40 aggregates to rat hippocampus to model amyloidogenic processes in 20 days only in neocortex and hippocampus (significant increase in concentration of A β 42 by 36% in neocortex and by 27% in hippocampus) while in olfactory bulbs, the concentration of A β 42 did not change (Fig. 1). 10 days of nasal therapy with liposome miR-101, started in 10 days after establishing experimental AD model, normalized A β 42 levels in target regions of rat brains, compared to empty liposomes. Thus, concentration of toxic endogenous A β 42 decreased by 33% in neocortex and by 15% in hippocampus. No changes were seen in olfactory bulbs.

These results are in line with previous findings. According to [43–44], miR-101 negatively regulated A β PP expression and accumulation of A β in neocortex, and its function decreased in patients with AD. A few authors link that to single-nucleotide polymorphism in 3'UTR region of A β PP gene [45]. There is now a body of evidence that miR-101 regulates the level of A β PP in cell cultures, particularly in hippocampal neurons [46–47].

Considering that A β PP and A β are the main factors of Alzheimer's disease pathogenesis, we suggest inhibiting the expression of A β PP to mitigate the pathological processes underlying amyloidosis. Consequently, miR-101 may become a new tool for therapeutic modulation of A β PP levels. It is possible that

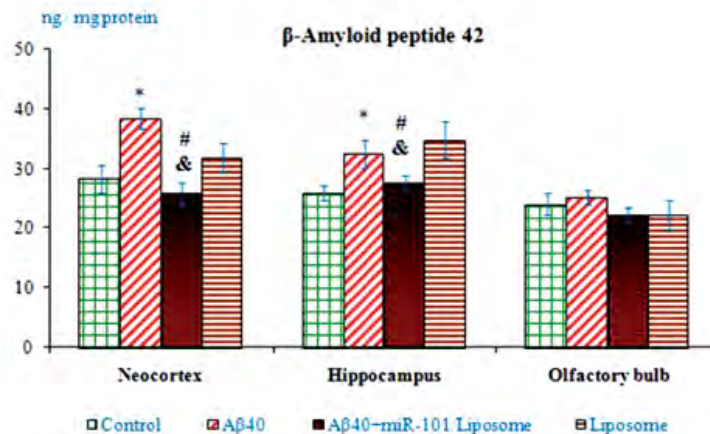


Fig. 1. Level of Aβ42 in neocortex, hippocampus, and olfactory bulbs in rats with experimental model of Alzheimer's disease, nasally treated with liposome miR-101 for 10 days
 Hereinafter * — $P \leq 0.05$ compared to control; # — $P \leq 0.05$ compared to AD model (Aβ40 group); & — $P \leq 0.05$ compared to therapy with empty liposomes (Liposome group)

either directly delivering miR-101 to CNS, or regulating its endogenous expression, should reduce AβPP levels in brains of patients. In [47] it was shown that miR-101 is expressed from two independent genomic loci contained in the intergenic regions on chromosome 1 and chromosome 9. The promoter elements regulating the transcription of miR-101 have not been sufficiently studied, and only now their detailed research is underway. Therefore, nasal therapy of miR-101 in liposomal form may be promising for the treatment of patients with Alzheimer's disease.

2. Anti-inflammatory effect of miR-101

Using an experimental AD model, it was shown that exogenous Aβ40 induces anti-inflammatory processes in neocortex and hippocampus (possible increase of total studied cytokines by 16–18% in neocortex and inflammatory cytokines TNFα and IL-6 by 14% in hippocampus). In olfactory bulbs, cytokine levels did not change significantly (Fig. 2, A, B, C).

Ten days of nasal administration of miR-101 in liposomal form decreased level of IL-6 by 23% in neocortex and by 19% in hippocampus, which was statistically significant compared to AD model and therapy with empty liposomes (Fig. 2, A). Significant decrease of TNFα levels by 12% under effect of miR-101 was seen only in animal hippocampus (Fig. 2, B). Unexpectedly, concentration of TNFα decreased in olfactory bulbs of rats with AD model after nasal treatment with empty liposomes (by 13%) and with liposomes containing miR-101 (by 10%). The levels

of IL-10 did not change significantly under influence of miR-101 in any of the brain region in AD model rats (Fig. 2, C). Notably, treatment with liposomal miR-101 strongly affected the levels of IL-6 (Fig. 2). This can be caused by experimental conditions. At the 20th day of experiment, TNFα, formed and secreted early under treatment, becomes less prominent in the neural inflammation compared to second generation cytokine such as IL-6 [48].

Comparing our data to the previous findings on anti-inflammatory effect of curcumin in liposomes under similar experimental conditions [37], it should be noted that polyphenol has higher anti-cytokine potential than miR-101. Anti-cytokine potential of curcumin can be explained by the direct effect it has on the levels of cytokine genes induction, and its indirect influence on the Aβ level in animal CNS. MiR-101 targets the mRNA from which AβPP is translated.

Decreasing concentration of inflammatory cytokines (IL-6 and TNFα) under effect of miR-101 is, in our opinion, not a direct effect. It is related to the falling levels of the toxic endogenous Aβ42 in neocortex and hippocampus (brain regions that are responsible for memory and studying). However, a number of authors assume that miR-101 may have a possible direct influence by decreasing the induced levels of inflammatory cytokines [49], by increasing IL-6 production in response to transfection of cells with miR-101 [50], or in case of excessive expression in LPS-activated macrophages [51].

Thus, the feasibility of combining miR-101 and curcumin in a single liposomal

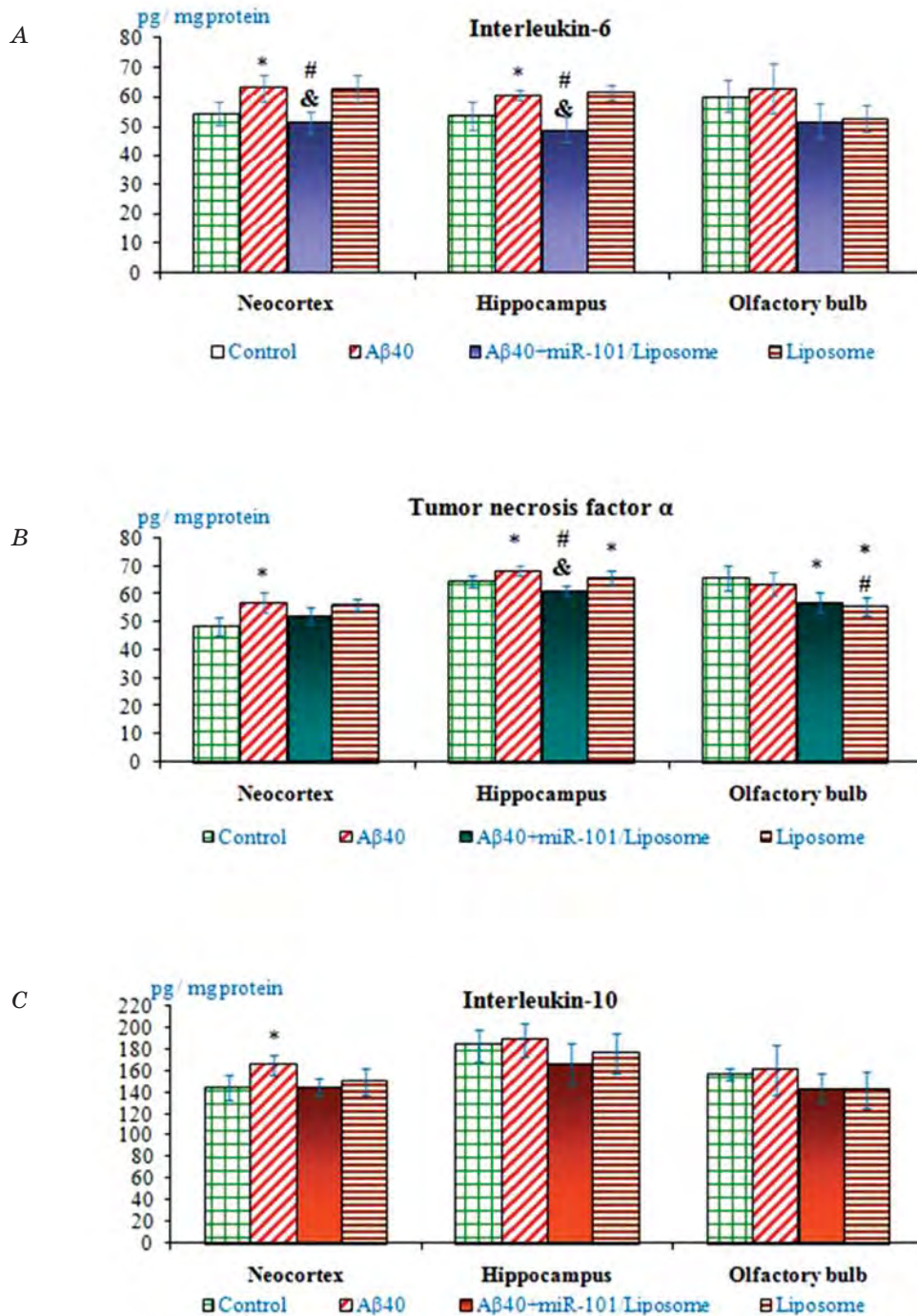


Fig. 2. Concentration of IL-6 (A), TNF α (B) and IL-10 (C) in AD model rats treated nasally with miR-101 in liposomes for 10 days

preparation should be considered to simultaneously eliminate the excess synthesis of A β PP with the formation of toxic aggregates of β -amyloid peptides and chronic neuroinflammation.

Thus, nasal therapy with miR-101 in liposomal form caused significant anti-amyloidogenic effect (normalization of A β 42

levels in neocortex and hippocampus in rat brains with AD model).

Anti-inflammatory effect of miR-101 in liposomal form caused decrease in concentrations of inflammatory cytokines (IL-6 and TNF α in neocortex and TNF α in hippocampus of animals with experimental AD) due to decreased level of toxic endogenous A β 42.

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АНТИАМИЛОДОГЕННА ДІЯ miR-101 ЗА ЕКСПЕРИМЕНТАЛЬНОЇ ХВОРОБИ АЛЬЦГЕЙМЕРА

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Метою дослідження було визначення впливу miR-101 на рівень β-амілоїдного пептиду й активацію системи цитокінів у відділах головного мозку тварин за експериментальної моделі хвороби Альцгеймера. MiR-101 є ключовим оператором функції мРНК для протеїну попередника β-амілоїдного пептиду шляхом її деактивації і здатна пригнічувати його синтез та амілоїдогенний процесинг. Щурам-самцям пізнього зрілого віку інтрагіпокампально одноразово унілатерально вводили агрегати β-амілоїдного пептиду 40 у дозі 15 нмоль. Через 10 діб розпочинали назально вводити ліпосомальну форму miR-101 або пусті ліпосоми. Після 10 діб щоденної терапії у неокортексі, гіпокампі та нюхових цибулинах визначали рівень токсичної ендогенної форми β-амілоїдного пептиду 42 й активність цитокінової системи за показниками фактора некрозу пухлини α, інтерлейкіну-6, інтерлейкіну-10. Встановлено, що екзогенні агрегати β-амілоїдного пептиду 40 моделюють у щурів амілоїдогенний і прозапальний стан через 20 діб лише у неокортексі та гіпокампі (достовірно збільшення концентрації β-амілоїдного пептиду 42 на 36% і цитокінів на 16–18% в неокортексі та β-амілоїдного пептиду 42 — на 27% і прозапальних цитокінів фактора некрозу пухлин α, інтерлейкіну-6 — на 14% у гіпокампі), проте не в нюхових цибулинах. Десятиденний курс назальної терапії ліпосомальною miR-101 нормалізував рівень β-амілоїдного пептиду 42 та цитокінів: у неокортексі концентрація ендогенного токсичного β-амілоїдного пептиду 42 зменшилася на 33%, у гіпокампі — на 15%, а прозапальних цитокінів — на 11–20%. Таким чином, назальна терапія miR-101 у ліпосомах зумовила достовірний антиамілоїдогенний ефект у щурів з моделлю хвороби Альцгеймера, тоді як її антизапальна дія передусім сприяла зниженню концентрації β-амілоїдного пептиду 42.

Ключові слова: miR-101, β-амілоїдний пептид, амілоїдоз, хвороба Альцгеймера.

АНТИАМИЛОДОГЕННОЕ ДЕЙСТВИЕ MiR-101 ПРИ ЭКСПЕРИМЕНТАЛЬНОЙ БОЛЕЗНИ АЛЬЦГЕЙМЕРА

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Целью исследования было определение эффекта miR-101 на уровень β-амилоидного пептида и активацию системы цитокинов в отделах головного мозга животных с экспериментальной моделью болезни Альцгеймера. MiR-101 является ключевым оператором функции мРНК для протеина предшественника β-амилоидного пептида путем ее деактивации и способна подавлять его синтез и амилоидогенный процессинг. Крысам-самцам позднего зрелого возраста интрагиппокампально одноразово унілатерально вводили агрегаты β-амилоидного пептида 40 в дозе 15 нмоль. Через 10 суток начинали назально вводить липосомальную форму miR-101 или пустые липосомы. После 10 суток ежедневной терапии в неокортексе, гиппокампе и обонятельных луковицах определяли уровень токсической эндогенной формы β-амилоидного пептида 42 и активности цитокінової системи по показателям фактора некроза опухоли α, интерлейкина-6, интерлейкина-10. Установлено, что экзогенные агрегаты β-амилоидного пептида 40 моделируют у крыс амилоидогенную и провоспалительную ситуацию через 20 суток только в неокортексе и гиппокампе (достоверное увеличение концентрации β-амилоидного пептида 42 на 36% и цитокинов на 16–18% в неокортексе и β-амилоидного пептида 42 — на 27% и провоспалительных цитокинов фактора некроза опухоли α, интерлейкина-6 — на 14% в гиппокампе), однако не в обонятельных луковицах. Десятидневный курс назальной терапии липосомальной miR-101 нормализовал уровень β-амилоидного пептида 42 и цитокинов: в неокортексе концентрация эндогенного токсического β-амилоидного пептида 42 уменьшилась на 33%, в гиппокампе — на 15%, а провоспалительных цитокинов — на 11–20%. Таким образом, назальная терапия miR-101 в липосомах обусловила достоверный антиамилоидогенный эффект у крыс с моделью болезни Альцгеймера, в то время как ее противовоспалительное действие прежде всего способствовало снижению концентрации β-амилоидного пептида 42.

Ключевые слова: miR-101, β-амилоидный пептид, амилоидоз, болезнь Альцгеймера.

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