INFLUENCE OF CURCUMIN ON CYTOKINES CONTENT AND ANGIOTENSIN-CONVERTING ACTIVITY UNDER INTRAHIPPOCAMPUS ADMINISTRATION OF β-AMYLOID PEPTIDE IN RATS

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The purpose of this study was to investigate the effect of curcumin on cytokine response and angiotensin-converting activity in terms of intrahippocampus administration of β-amyloid peptide in rats. Animals with experimental model of Alzheimer's disease received nasal therapy aqueous solution of curcumin. Recorded concentration of cytokines (interleukin-1β, interleukin-6, interleukin-10, tumor necrosis factor-α) and angiotensin-converting activity in the parts of the brain (cerebral cortex and hippocampus) and blood serum, as well as indicators of conditional reflex escapers action. Install a reduction in cytokine response intrahippocampus administering Aβ42_Human and angiotensin-converting activity inhibition in the brain but not in the blood serum of animals under the action of curcumin. Recorded improvement in cognitive performance in rats with a model of Alzheimer's disease as a result of curcumin therapy. Nasal treatment with an aqueous solution of curcumin has anti-inflammatory effect in the targeted parts of the brain (cerebral cortex and hippocampus), but inhibits angiotensin-converting activity.

Curcumin (diferuloylmethane) is a natural polyphenol which manifests compound effect on the body homeostasis; it negatively influences the NF-kB and AP-1 transcription factors, suppresses expression of cyclooxygenase-2, lipoxygenase, NO-synthase, matrix metalloproteinase-9, urokinase of plasminogen type activator, tumor necrosis factor (TNF), chemokines, molecules of cell adhesion and cycline D1; it inhibits expression of growth factor receptors and activity of stress-associated proteinkinase (JNK), protein tyrosine kinases, as well as other protein serine/threonine kinases [1−3]. Curcumin also acts as inhibitor of DNA-methyltransferase, therefore it is regarded as DNA hypomethylating agent. It establishes balance between activity of histone acetyltransferase and histone deacetyltransferase thus influencing the expression of certain genes. At last, curcumin modulates activity of micro-RNAs and their numerous target genes [4−5]. The above-mentioned effects of curcumin are accumulated in its anti-oxidation, anti-inflammation, anti-tumor and anti-amyloidogenic properties [6−11].

Considered recently as one of probable Alzheimer's disease (AD) factors is aggregation of β-amyloid peptide (Aβ) to fibrils or deposits as the main pathogenetic event [12−15]. In particular, several studies showed that Aβ is essentially accumulated in the brain areas (hippocampus and cortex), which conduce to obtaining and processing of information, and memory [16−18]. This peptide is formed during amyloidogenic processing of amyloid protein precursor (APP) [19−21]. In a non-amyloidogenic way the full-size APP is decomposed by α- and γ-secretases within Golgi apparatus and plasmatic membrane without forming the β-amyloid peptide. Back internalization of a certain part of APP from the plasmatic membrane ant its transport towards the late endosomes results in β- and γ-secretase-associated separation of Aβ isoforms, 38 to 43 amino acid residues long [22]. The role of switch between non-amylodogenic and amyloidogenic ways of APP processing is

played by various factors: excess of APP or the rate of its phosphorylating, intensity of expression of SORL1 gene of the receptor to apolipoprotein E, presence of mutations in APP and presenilins [23–27].

Endogenic Aβ is a critical player in the synaptic plasticity memory of the central nervous system in norm [28–30]. It was shown that at low (picomolar) concentrations Aβ may act as a trophic signal and modulator of the synaptic activity [31–33]. Besides, Aβ may function as anti-oxidant due to its capability to bond the oxide-reduced metals, and as chelating agent [34–36]. Aβ is important for development of neurons, their plasticity and survival due to its integral interaction with membranes, support of structural integrity of hematoencelphalic barrier (HEB); it has anti-microbe properties and modulates the transport of Ca^{2+} through membranes [37–41].

At high (nanomolar and micromolar) concentrations of Aβ, the neurotoxic aggregates are formed: oligomers or fibriles, amyloidosis and cell death [42–44]. The mechanism due to which the β-amyloid peptide causes damage and death of neurons lies in generations of the oxygen active forms in the course of own aggregation. At the same time on the neuron membranes, peroxide oxidation of lipids is activated and adenosine triphosphatase function is impaired. As a result, Aβ conduces to depolarization of the synaptic membranes, excess input of Ca^{2+} and mitochondrial insufficiency [45–47]. All those processes are concurrent with non-specific inflammation response which goes chronic, and induce APP synthesis and its processing in terms of amyloidogenic scenario [48–50].

The approaches to amyloidosis therapy of Alzheimer's disease which are concentrated on suppression of production and aggregation of Aβ [51–53] or symptomatic therapy [54–58] are low-efficient, therefore correction of the chronic inflammation which provokes excess and aggregation of Aβ, may have positive effect. It was demonstrated that the inflammation process at AD is accompanied with increase in peripheral concentration of cytokines — interleukin-1β (IL-1β), interlukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α), and higher levels in transforming growth factor-β (TGF-β) in spinal liquid [60]. On the other side, the cytokines, as well as Aβ, are mediators of congenital immunity [59, 39]. They implement their effect through the receptor activation of intracellular signals, which results in translocation of NFκB to the nucleus and activation of protein synthesis

de novo [60–61]. However, the existing anti-cytokine therapy is not efficient for amyloidosis, except for anti-inflammation effect IL-10 [62–63]. The Aβ aggregation process is due to the impairment of the balance between its production and degradation. One of the systems supporting the low Aβ level in tissues is zinc metalloproteinases [64–65]. Belonging to them is also the angiotensin-converting enzyme (ACE), (КФ 3.4.15.1), which is involved in regulation of arterial tension, exchange of neuropeptides, protection and immunity functions of the body [66–68]. This enzyme (mainly its C-domain) separates the C-end dipeptides from oligopeptides of various structure which have a free carboxyl group. But ACE react with Aβ only by N-domain and decomposes the peptide bonds R5–H6 or D7–S8 [68]. ACE is a I type integral membrane glycoprotein which is released to blood circulation by zinc metalloesterase at the speed of 2% per hour, therefore it functions both in bonded and dissolved form. The conclusions of the studies of ACE1 gene polymorphism and its inhibitors state that decrease of ACE activity is associated with AD risk and accumulation of Aβ [69–70].

The purpose of this study was to investigate the effect of curcumin on cytokine response and angiotensin-converting activity in terms of intrahippocampus administration of β-amyloid peptide in rats.

Materials and Methods

Study Design
The study involved 30 mail mature rats weighing 200 to 250 grams. All the animals were kept at 12-hour light-dark cycle, standard feed for rodents and tap water. Experimental protocols complied with the rules of the European Convention for Protection of Vertebrate Animals used in experiments and for other scientific purposes (Strasbourg, 1986).

The rats were randomly distributed between 5 groups (6 animals each). The reference group included the intact animals. Group 1 — the rats 1 month after intrahippocampal injection of Aβ42_Human (Human Amyloid β Protein Fragment 1-42, Sigma-Aldrich, USA) — experimental model of AD; Group 2 — false-acts animals; Group 3 — the rats with experimental model of AD, which daily received the nasal therapy with aqueous solution of curcumin (Sigma-Aldrich, USA) for 1 month and Group 4 — the animals with experimental model of AD which daily received

the nasal therapy of the solvent (bidistilled water) for 1 month.

Cognitive Tests

Preliminarily, for 20 days in all the rats the conditioned reflex reaction on the basis of non-conditioned reflex elimination was formed [71]. As positive result were considered the infallible conditioned reflex responses to the sound of metronome. Next to the portion of the positive responses (in per cent), registered in the study was the duration of the conditioned reflex reaction elimination latent period in seconds. The animals of all the groups were tested for these values of conditioned reflex reaction elimination after they were formed the AD experimental model and curcumin or solvent therapy respectively.

Experimental Model of Alzheimer's Disease and Curcumin Therapy

Aβ42_Human solved in bidistilled water was aggregated for 24 hours at 37 °C. Large rough conglomerates of Aβ42_Human were dispersed, using the ultrasonic homogenizer (Musson-1, Russia) for 5 min and sterilized immediately before injection. The effect of β-amyloid peptide 42_Human in homoaggregate form was studied one month after its single injection in the dosage of 15 nM Aβ42_Human (65 micrograms) to the brain hippocampus of the rats. The volume of the solution: 10 microliters per animal. The stereotaxic coordinates of the left hippocampus were determined by the map of the rat brain [72], which corresponds to the distance from the point of intersection of the sagittal seam with bregma (zero point): distally — 2 mm, laterally — 2 mm and in depth — 3.5 mm. Stereotaxic operations in the investigated animals ran under general narcosis using intraperitoneal injections of thiopental, 50 mg/kg of body mass.

Since curcumin has low solubility in water, its concentrated solution was first prepared in 96% ethanol. Curcumin remained stable in ethanol at the room temperature for three weeks but degraded fast in water at neutral or weak basic pH [73]. Therefore the outgoing curcumin solution was dissolved in the bidistilled water to 0.7 g/l immediately before the nasal injection into the rats in the dosage of 3.5 µg/animal.

After the processing was finished the animals were decapitated. The samples of the cerebral cortex and hippocampus were frozen and stored for further measurement. The blood was taken and centrifuged at 1000g (OPN-3, Russia) for 20 min. The serum was collected, frozen and stored. The tissues of hippocampus and cerebral cortex were homogenized in Tris buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5), centrifuged at 14,000g (RS-6, Russia) for 5 min and then the supernatant was collected.

Immunity-Enzyme Analysis of Cytokines

The samples of hippocampus supernatant and cerebral cortex and blood serum were used to determine cytokines by the immunity-enzyme analysis (IEA) in accordance with the instructions (Rat ELISA Kits Invitrogen BCM DIAGNOSTICS, USA) for IL-1β, IL-6, IL-10 and TNF-α. The optical density was read out by GBG Stat FAX 2100 (USA) microplate analyzer at 450 nm with wavelength correction (630 nm). The data of the Immunity Enzyme Analysis (µg/l cytokins) were recalculated to the general protein or expressed in ng/l blood serum. In the figures the obtained data are represented in percentage to the reference group levels. The concentration of the general protein was determined by the Lowry method [74].

Kinetic Test of ACE Activity

ACE activity was determined by kinetic method [75]. Used as substrate was the FAPGG short peptide, from which under action of ACE GG dipeptide was separated and transformed into the hippuric acid. Decrease of the sample optical density at 10 min incubation and T = 37 °C was measured at the wavelength of 340 nm. ACE activity (E_{ACE}) was calculated using the following equation:

$$E_{ACE} = \frac{\Delta A_{sample}}{\Delta A_{calibrator\ hippuric\ acid}} E_{calibrator} \,,$$

where ΔA — decrease of optical density at 10 min incubation and T = 37 °C;

$$E_{calibrator} = 82,1$$
(protocol BÜHLMANN ACE colometric kit, Switzerland).

ACE activity was expressed in the activity units (U/l), which corresponds to the quantity of the ACE enzyme which separates 1 µM of hippuric acid at 37 °C per 1 min per liter for blood serum and per mg protein for the brain areas (cerebral cortex and hippocampus).

Statistical Processing of the Study Results

The obtained results were statistically processed, the average values and standard

deviations being calculated. The statistical analysis of differences was calculated using Student *t*-test comparing the groups 1, 2, 3 and 4, as well as comparing to the reference levels. The values of $P < 0{,}05$ were considered as significant.

Results and Discussion

Specificity of cytokine response at chronic neuroinflammation from intrahippocampal injection of β-amyloid peptide 42_Human (experimental model of AD) in rats

The animals with the experimental model of AD (Group 1) did not manifest any essential differences in the concentration of inflammation cytokines in blood serum, compared to the reference group. Table 1 shows that only the concentration of anti-inflammation IL-10 in serum is lower by 54%. The similar trends were registered in the group 2 (false-acts animals). Therefore, the level of the investigated cytokines in the blood serum does not reflect any specific effect of β-amyloid peptide 42_Human in the brain of the rats and represents the result of intracerebral intervention.

The study of cytokines in the cerebral cortex of the rats with the model of Alzheimer's disease (group 1) showed increase in the levels of IL-1β (by 109%) and IL-6 (by 54%) compared to the respective levels of the intact animals (reference), and the increase in concentrations of IL-6 (by 29%) and decrease of IL-10 (by 31%) compared to the false-acts animals (Fig. 1).

The content of TNF-α in the cerebral cortex and hippocampus of the rats in the groups 1 and 2 was not different from the reference levels and between each other.

The hippocampal levels of IL-1β and IL-10 in the rats with experimental model of AD were higher compared to the reference group (by 221% and 111%, respectively) and group 2 (by 110% and 78%, respectively). In this part of the brain the IL-6 concentration in the
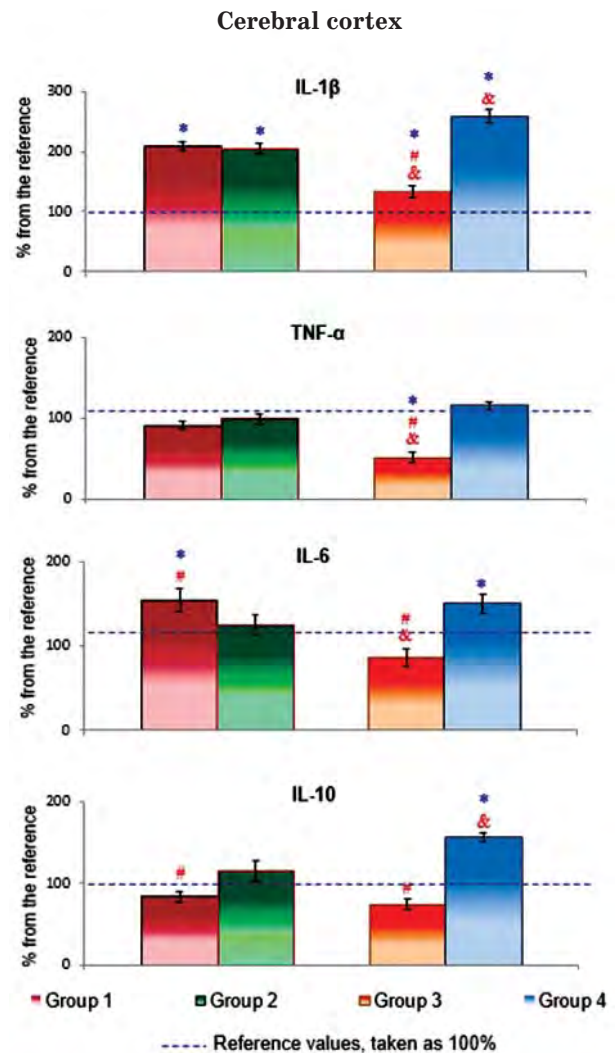

Cerebral cortex

Fig. 1. Effect of Aβ42_Human homoaggregates and curcumin therapy on the level of cytokines (IL-1β, TNF-α, IL-6, IL-10) in the brain cerebral cortex of the rats

The results are given in% from the reference as $M \pm m$. * — $P \leq 0.05$ compared to the reference; # — $P \leq 0.05$ at comparison of the groups 1 and 2 and 3 and 4, respectively; & — $P \leq 0.05$ compared to the group 1 (experimental model of AD).

Table 1. **Effect of β-amyloid peptide 42_Human homoaggregates and curcumin on IL-1β, TNF-α, IL-6 and IL-10 in blood serum of the investigated rats**

| Cytokine	Reference (ng/l)	Group 1 (ng/l)	Group 2 (ng/l)	Group 3 (ng/l)	Group 4 (ng/l)
IL-1β	17.2±1.2	16.8±1.1	15.7±0.6	20.9±2.3*#&	16.2±1.2
TNF-α	7.9±0.8	9.5±0.6*	10.7±1.0*	10.3±1.4*#	13.0±1.2*&
IL-6	4.8±1.0	3.7±0.6	5.3±1.1	1.8±0.5*&	1.8±0.3*&
IL-10	3.9±0.4	1.8±0.2*	1.3±0.2*#	4.0±0.5#&	1.6±0.2*

The results are presented as $M \pm m$;

* — $P \leq 0.05$ compared to reference;

— $P \leq 0.05$ at comparison of the groups 1 and 2 and 3 and 4, respectively;

& — $P \leq 0.05$ compared to group 1 (experimental model of AD).
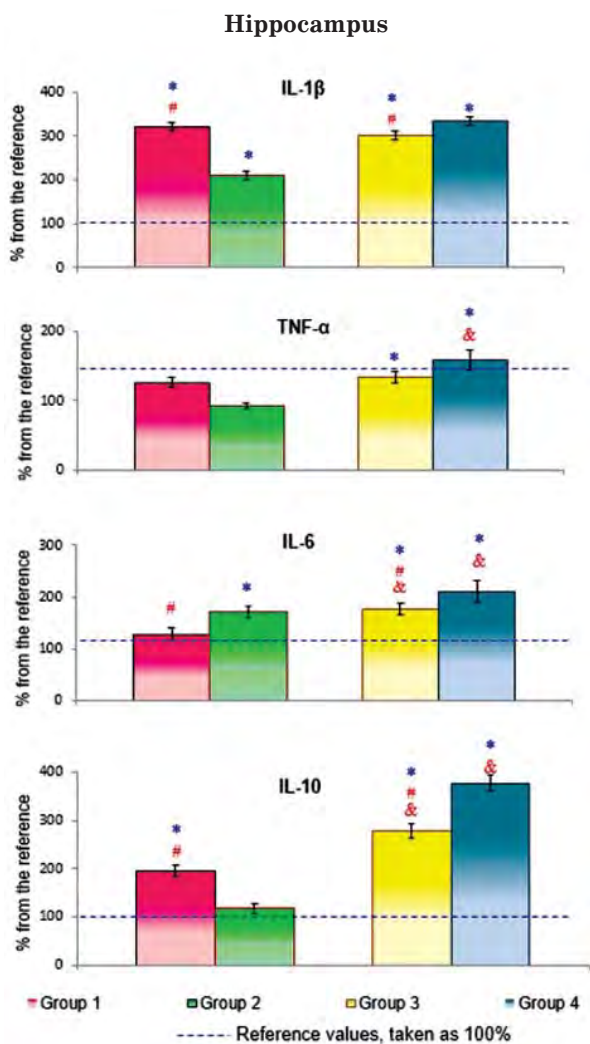
Hippocampus

Fig. 2. Effect of Aβ42_Human homoaggregates and curcumin therapy on the level of cytokines (IL-1β, TNF-α, IL-6, IL-10) in the brain hippocampus of the rats

The results are given in% from the reference as $M \pm m$. * — $P \le 0.05$ compared to the reference; # — $P \le 0.05$ at comparison of the groups 1 and 2 and 3 and 4, respectively; & — $P \le 0.05$ compared to the group 1 (experimental model of AD).

rats from group 1 was not different from the values of the intact animals, but was lower by 44% against the IL-6 concentration in the false-acts rats (Fig. 2). These data show that Aβ42_Human homoaggregates in the hippocampus of the rats with AD experimental model caused chronic neuroinflammation specifically and mainly in the place of injection. But manifested also in the brain cerebral cortex of these rats was inflammation activation, although to lower extent. This result confirms the conclusion of the study [76], where it was shown that Aβ40_Human homoaggregates injected into the brain cerebral cortex of the rats cause higher cytokine response just in the area of the injection.

Increase in ACE activity and memory suppression induced by Aβ42_Human

Table 2 shows the data about increase in ACE activity in the hippocampus and blood serum at intracerebral action of Aβ42_Human.

This is explained first of all by the induction of ACE synthesis with local excess of the substrate (β-amyloid peptide). Probability of this assumption is shown by the data of our studies [76] and the studies [77−78].

Intrahippocampal injection of the Aβ42_Human homoaggregates caused suppression of the elimination conditioned reflex reaction in the rats of group 1. The study of the cognitive capabilities and memory showed decrease in the portion of the positive responses and increase in the latent period for these animals compared to the rats of the reference group (Fig. 3). The portion of the positive responses in the rats with AD experimental model was not different from the level of the false-acts animals, which describes the consequences of the intracerebral intervention.

The obtained results showed presence of the cytokine system activation in the brain of the rats with AD experimental model (Fig. 1, 2). These data are in agreement with

Table 2. **Effect of β-amyloid peptide 42_Human homoaggregates and curcumin on ACE activity in the blood serum and brain sections of the rats**

Group \ Item	Blood serum U/l	Frontal cortex U/mg protein	Hippocampus U/mg protein
Reference	3.8±0.2	33.3±5.2	30.9±3.3
Group 1	11.5±1.0*&	36.1±0.8&	46.2±2.8*&
Group 2	4.2±0.3	29.3±4.1	36.5±5.6
Group 3	6.3±0.8*	18.5±2.1*&	22.2±1.7*&
Group 4	5.5±0.6*	31.8±4.4	81.8±8.3*

* — $P \le 0,05$ compared to the reference;
& — $P \le 0,05$ at comparison of the groups 1 and 2 and 3 and 4, respectively.
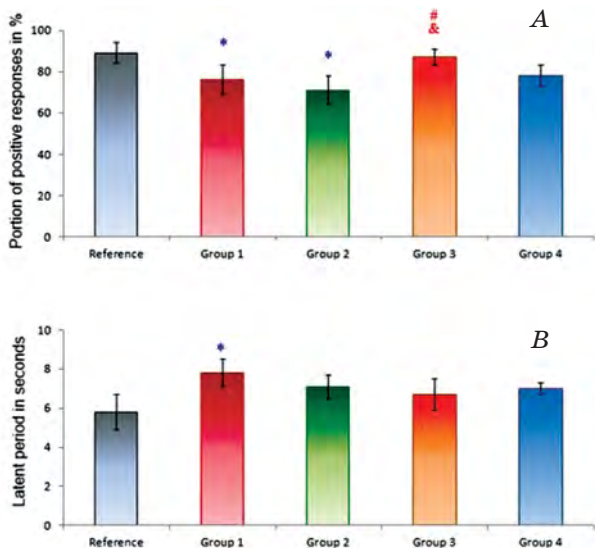
Fig. 3. **Changes in the portion of positive responses (*A*) and latent periods (*B*) during action of the β-amyloid peptide 42_Human homoaggregates and curcumin therapy in the rats**

The results are given as $M \pm m$. * — $P \leq 0.05$ compared to the reference; # — $P \leq 0.05$ at comparison of the groups 1 and 2 and 3 and 4, respectively; & — $P \leq 0.05$ compared to group 1 (AD experimental model).

other investigations of the neuroinflammation activation by Aβ aggregates [79–84]. Aβ deposits are responsible for microglia activation [79]. Aβ conduces to increase in inflammatory response to NF-κB stimulation, which is involved in the regulation of extracellular signal-regulated kinase (ERK) and mitogen-activated proteinkinase (МАРК), leading to production of cytokines and chemokines [80]. Toll-like receptors (TLR), similar to the receptors of inflammatory interleukins IL-1 and TNF, are important for regulation of microglia response to Aβ. Modification of the inflammatory state of microglia/ macrophage plays prominent role in the course of amyloidosis [81].

Chronic neuroinflammation induced by injection of Aβ42_Human into hippocampus, caused changes in the levels of TNF-α and IL-10 in blood serum (Table 1), and lower cognitive capabilities and memory in the rats (Fig. 3). So, hyperproduction of cytokines may play the role of implementing mechanism at the first stages of amyloidosis and dementia. Further, the detected increase in ACE activity in response to the injection of β-amyloid peptide into hippocampus did not prevent from lower values of the cognitive tests with the investigated animals.

Dualism of curcumin effects in the rats with AD experimental model

Daily curcumin therapy of the rats with AD model for 1 month caused increase in the levels of inflammatory cytokines: IL-1β (by 22%) and TNF-α (and 30%) and decrease in the level of ambivalent IL-6 (by 38%) in blood serum compared to the reference values (Table 1). In group 4 (nasal injection of bidistillate instead of curcumin solution into the rats with AD experimental model) the IL-1β content remained unchanged but dynamics of TNF-α and IL-6 concentrations coincided with group 3. Curcumin specifically restored the concentration of anti-inflammatory IL-10 in blood serum of the rats, contrary to the effect of bidistilled water. It may be noted that in case of curcumin therapy the cytokine response is activated in the blood circulation of the investigated rats.

The curcumin effect in the cerebral cortex gave specific inhibition of cytokines (Fig. 1). Under action of curcumin the levels of IL-1β and IL-6 were normalized; TNF-α level dropped by 49%, compared to the reference; IL-10 concentration did not change but was not different from the reference. Observed in group 2 was further aggravation of the neuroinflammatory process induced by intrahippocampal injection of β-amyloid peptide 42_Human. Concentrations of IL-1β and IL-10 in this brain part increased by 50% and 73%, respectively, during one month of the bidistillate nasal therapy.

In hippocampus of the animals curcumin effect on cytokine values had the similar trend (Fig. 2). But not a single cytokine normalized its concentration, on the contrary the level of IL-6 (by 49%) and IL-10 (by 83%) increased compared to the values at the beginning of the month. But when the hippocampus cytokine values are compared in the groups 3 and 4, the specific suppression becomes understandable concerning the levels of IL-1β (by 33%), TNF-α (by 24%), IL-6 (by 34%) and IL-10 (by 99%) caused by curcumin effect. The detected anti-inflammatory activity of curcumin resulted in restoration of the memory values, in particular the portion of the positive responses of the animals (Fig. 3).

Therefore the previous assumption that curcumin may be efficient anti-inflammatory factor in case of exogenous β-amyloid peptide found confirmation in the above-mentioned experimental data. This natural polyphenol blocks activation of the inflammatory factor of NF-κB transcription, suppressing phosphorylating and degradation of IκBα

(NF-κB inhibitor). Since the curcumin effect lies in inhibition of activation of IB kinase (IKK), required for NF-κB activation [85−87], just this fact may explain the detected anti-cytokine effect of curcumin in the investigated animals (Fig. 1−2).

The suppressive action of curcumin on ACE activity in hippocampus (by 3.7 times) and cerebral cortex (by 1.7 times) of the brain and absence of changes in the blood serum of the rats were shown (Table 2). The mechanism of curcumin effect on ACE activity is substantiated by suppressive influence of this polyphenol on the expression of the enzyme gene [88].

It is in the presence of such contrary effects that dualism of curcumin influence on the Alzheimer's disease experimental model in the rats lies.

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ВПЛИВ КУРКУМІНУ НА ВМІСТ ЦИТОКІНІВ І АНГІОТЕНЗИН-ПЕРЕТВОРЮВАЛЬНУ АКТИВНІСТЬ ЗА ІНТРАГІПОКАМПАЛЬНОГО ВВЕДЕННЯ ЩУРАМ β-АМІЛОЇДНОГО ПЕПТИДУ

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Мета дослідження — вивчення впливу куркуміну на вміст цитокінів і ангіотензин-перетворювальну активність за умов інтрагіпокампального введення щурам β-аміло їд ного пептиду. У тварин з експериментальною моделлю хвороби Альцгеймера застосовували назальну терапію водним розчином курку міну. Реєстрували концентрацію цитокінів (інтерлейкіну-1β, інтерлейкіну-6, інтерлейкіну-10, фактора некрозу пухлин α) і ангіотензинперетворювальну активність у відділах головного мозку (лобно-фронтальна кора і гіпокамп) та сироватці крові, а також показники умовно-рефлекторної реакції уникнення. За дії куркуміну встановлено зниження вмісту цитокінів на інтрагіпокампальне введення β-аміло їдного пептиду 42_Human і пригнічення ангіотензинперетворювальної активності в головному мозку, але не в сироватці крові. У результаті куркумінової терапії відзначено поліпшення когнітивних показників у щурів із хворобою Альцгеймера. Назальна терапія водним розчином куркуміну справляє антиза пальний ефект у цільових відділах головного мозку (лобно-фронтальна кора і гіпокамп) та пригнічує ангіотензинперетворювальну активність.

Ключові слова: куркумін, β-аміло їдний пептид, цитокіни, ангіотензинперетворювальний ензим, хвороба Альцгеймера.