

EFFECT OF CURCUMIN LIPOSOMAL FORM ON ANGIOTENSIN CONVERTING ACTIVITY, CYTOKINES AND COGNITIVE CHARACTERISTICS OF THE RATS WITH ALZHEIMER'S DISEASE MODEL

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The purpose of the study was the investigation of curcumin liposome form effect on angiotensin-converting enzyme activity, cytokines and mnesic features of rats with experimental model of Alzheimer's disease. In the animals with intrahippocampal injection of A β 42_Human, nasal therapy with curcumin liposome form was used. Cytokine concentration and angiotensin converting enzyme activity in brain regions (cerebral cortex and hippocampus) and in blood serum as well as indicators of conditioned avoidance response were registered. It was found that as a result of curcumin therapy the rats with Alzheimer's disease had suppressed cytokine and angiotensin converting enzyme activities and recovered mnesic indices. Nasal therapy with curcumin liposome form gave reduction of angiotensin-converting enzyme activity and anti-cytokine effect in the target regions of the brain (cerebral cortex and hippocampus), which helped the rats mnesic features and memory recovery.

Key words: curcumin, liposomes, β -amyloid peptide, cytokines, angiotensin converting enzyme.

The hitherto used approaches to treatment of dementia and amyloidosis in the case of Alzheimer's disease (AD) concentrated on suppression of β -amyloid peptide (A β) production and aggregation or on symptomatic therapy are ineffective [1–5], therefore correction of chronic inflammation provoked by amyloidosis will have positive effect. The mechanism by which A β causes the damage and death of neurons is generation of oxygen active forms in the course of own aggregation. At the same time neuron membranes lipids peroxidation is activated and ATPases function is deteriorated. As a result A β conduces to depolarization of synaptic membranes, excessive ingress of Ca²⁺ and mitochondrial insufficiency [6–8]. All these processes are concurrent with non-specific inflammatory reaction which is transformed into chronic form and induces synthesis of A β protein precursor (A β PP) and its processing pursuant to amyloidogenic scenario [9–11]. It is shown that the inflammatory process in case

of AD is characterized by increased peripheral concentrations of anti-inflammatory cytokines and higher TGF- β levels in spinal cord liquid [12]. On the other side, cytokines, similar to A β , are mediators of inborn immunity [13, 14]. Their effect comes up through receptor activation of intracellular signals, which results in translocation of nuclear factor (NF κ B) towards nucleus and activation of protein synthesis *de novo* [12]. In the end, the existing anti-cytokine therapy poorly represented itself for amyloidosis, except for anti-inflammatory effect of IL-10 [15]; although AD risk is lower in patients who take non-steroid anti-inflammatory preparations [16, 17]. Therefore we assumed that curcumin (CUA) with its anti-inflammatory properties may have essential therapeutic effect against A β -induced neurotoxicity and cognitive deficiency.

It is found that natural polyphenol CUA regulates NF κ B, AP-1 transcription factors; suppresses expression of cyclooxygenase-2,

lipoygenase, NO-synthase, matrix metalloproteinase-9, urokinase of plasminogen activator type, TNF, chemokines, cellular adhesion molecules and D1 cycline, inhibits expression of growth factor receptors and activity of JNK, protein tyrosine kinases as well as some other protein serine/threonine kinases [18, 19]. Curcumin also acts as inhibitor of DNA-methyltransferase therefore it is regarded as DNA hypomethylating agent. It establishes equilibrium between histone acetyltransferase and histone acetylase enzymes activity thus modulates expression of certain genes. At last CUA modulates activity of microRNA and their numerous target genes [20, 21]. Above-mentioned CUA effects are exhibited in its antioxidant, anti-inflammatory, anti-tumor and even anti-amyloidogenic properties [22–27]. The problem with curcumin usage, like with the other hydrophilic anions, lies only in the fact that it cannot enter the cell through plasmatic membrane on its own. Therefore it is reasonable to use nanocarriers, in particular, liposomes, as CUA carriers. Liposome advantages are obvious: prepared from natural phospholipids, they compared to other polymeric delivery systems, completely biodegrade in the body and are biocompatible [28].

β -Amyloid peptide aggregation process results in imbalance between its production and degradation. One of the systems, which maintain low $A\beta$ level in tissues, are zinc metalloproteinases [29]. Angiotensin-converting enzyme (ACE) [EC 3.4.15.1], which is involved in regulation of arterial blood pressure, neuropeptide exchange, immune responses of the body is also among them [30]. This enzyme (chiefly its C-domain) separates C-terminal dipeptides from oligopeptides of various structures which have a free carboxyl group. But ACE interacts with $A\beta$ exclusively by N-domain and decomposes Arg5–His6 or Asp7–Ser8 peptide bonds [30]. ACE is an integral membrane glycoprotein of the 1st type which is released to blood circulation by zinc metalloesterase at the rate of 2% per hour therefore this enzyme functions both in bonded and dissolved forms. According to the conclusions of ACE1 gene polymorphism and enzyme inhibitors studies it was found that ACE activity reduction is associated with AD risk and $A\beta$ accumulation [31].

The purpose of the study was investigation of curcumin liposome form effect on ACE activity, cytokines and mnemonic features of rats with experimental model of Alzheimer's disease.

Materials and Methods

The study involved 30 male rats of sexually mature age, with 200 to 250 g weight. All the animals were kept under controlled 12-hour light-darkness cycle with standard fodder for rodents and tap water. Experimental protocols were conducted in accordance with the rules of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986).

The rats were randomly distributed into 5 groups (6 animals in each). The reference group included intact animals; group 1 — the rats 1 month after intrahippocampal injection of A β 42_Human (experimental model of AD); group 2 — sham operated animals; group 3 — the rats with AD experimental model which received daily nasal therapy with curcumin liposome form for 1 month, and group 4 — animals with AD experimental model which received daily nasal therapy with empty liposomes also for 1 month.

Beforehand, during 20 days conditioned avoidance response was formed in the rats on the basis of unconditioned reflex [32]. Infallible conditioned responses to metronome sound were considered as positive result. Apart from positive response portion (in %), the study registered duration of latent period of conditioned avoidance response in seconds. Animals from all groups were tested in the conditioned avoidance response parameters after AD experimental model formation in them and nasal therapy with curcumin liposome form or empty liposomes.

A β 42_Human (Human Amyloid β Protein Fragment 1-42, Sigma-Aldrich), dissolved in bidistilled water was aggregated for 24 hours at 37 °C. A β 42_Human large size rough conglomerates were dispersed by ultrasound and sterilized immediately before injection. The effect of 42_Human β -amyloid peptide was studied 1 month after its single injection in the dose of 15 nM A β 42_Human to the hippocampus of the rats' brain. Solution volume was 10 μ l per animal. Stereotaxic coordinates of the left hippocampus were determined using the brain map of the rats [33], which corresponds to the distance from the intersection point of sagittal seam and bregma (zero point): distally — 2 mm, laterally — 2 mm and in depth — 3.5 mm. The stereotaxic operations in experimental animals were made under general anesthesia using intra-abdominal injections of thiopental (50 mg/kg of body weight).

To prepare liposomes with curcumin, lecithin/cholesterol was dissolved in the round-bottom flask at ratio 18:1 in 50% ethanol. After the lipid film was formed as a result of the solvent evaporation, 28.85 mM CUA in 5 ml of PBS buffer (10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4) was added and thoroughly mixed for suspension of liposomes with curcumin formation. The suspension of empty liposomes was prepared using the similar protocol but at the final stage PBS buffer without CUA was added. Both liposome suspensions were dissolved with PBS buffer to 0.7 g/l CUA immediately before nasal administration to the rats in the dose of 3.5 µg/animal. Daily nasal therapy of the rats with AD experimental model lasted for 1 month. Administration of liposome form curcumin by nasal method is determined by the fact that, unlike peripheral blood circulation, this is the shortest way to the target regions of the rat neocortex. It is known that after entering the body the dissolved curcumin is nearly unable to overcome the hematoencephalic barrier whereas its liposome form is actively and non-specifically entrapped by the formed elements of blood, which requires big doses of the preparation.

After the processing was finished the animals were decapitated. The assays of cerebral cortex and hippocampus were frozen and stored for further measurements. Blood was collected and centrifuged at 1000 g for 20 min. Serum was collected, frozen and stored. The tissues of hippocampus and fronto-coronal cortex were homogenized in Tris-buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5), centrifuged at 14000 g for 5 min producing supernatant.

In the supernatant assays of hippocampus, cerebral cortex and blood serum cytokines were identified using ELISA method in accordance with the protocol Rat ELISA Kit Invitrogen BCM DIAGNOSTICS, USA for interleukine-6 (IL-6), interleukine-10 (IL-10) and tumor necrosis factor α (TNF α). Assay absorption was read out using GBG Stat FAX 2100 (USA) microplate analyzer at 450 nm with wavelength correction at 630 nm. The ELISA data were recalculated into general protein for nerve tissue or expressed in ng/l for blood serum. Concentration of general protein was quantitated by Lowry method [34].

ACE activity was determined by kinetic method [35]. As a substrate the short FAPGG peptide was used, from which by ACE action GG dipeptide was separated and transformed into hippuric acid. Reduction in assay

absorption during 10 minute incubation at 37 °C was measured at 340 nm wavelength. Calculation was made using formula:

$$E_{ACE} = (\Delta A_{\text{assay}} / \Delta A_{\text{hippuric acid calibrator}}) \cdot E_{\text{calibrator}},$$

where ΔA — reduction of absorption during 10 min of incubation at 37 °C; $E_{\text{calibrator}} = 82.1$ (protocol B HLMANN ACE colometric kit, Switzerland). ACE activity was expressed in U/l, which corresponds to ACE enzyme quantity, which separates 1 µM hippuric acid at 37 °C per minute per liter for blood serum and per mg of protein for areas of the brain (fronto-coronal cortex and hippocampus).

The obtained results were statistically processed, average values and standard deviations were calculated. Statistical analysis of differences was made using *t*-test. Values at $P < 0.05$ were considered significant.

Results and Discussion

Fig. 1 presents data about increase in ACE activity in hippocampus (direct spot of A β 42_Human injection) and blood serum and absence of reliable changes in this parameter in cerebral cortex of the rats with AD experimental model. It is first of all due to ACE synthesis induction by one of the substrates local excess, namely A β [36]. Nasal therapy with CUA liposome form significantly reduced ACE activity compared to the effect of empty liposomes (Fig. 1) in both investigated brain sections of the rats and in blood serum.

On physiological level, the intrahippocampal injection of A β 42_Human brought suppression of conditioned avoidance response in the rats of group 1 (Fig. 2). Study of mnemonic features and memory showed reduction in the share of positive responses and increase of latent period in these animals compared to the reference group. The share of positive responses in the rats with AD experimental model was not different from that of in the sham operated animals, which is a consequence of intracranial intervention. Curcumin in liposomes was responsible for recovery of mnemonic features and memory parameters in the rats with AD experimental model, which was not the case when empty liposomes were used (Fig. 2).

The most substantial cytokine activation was registered in hippocampus of the animals with AD experimental model (Table 1): Concentration of TNF α increased by 26%, IL-6 — by 27% and IL-10 — by 95%, respectively. These data show that A β 42_

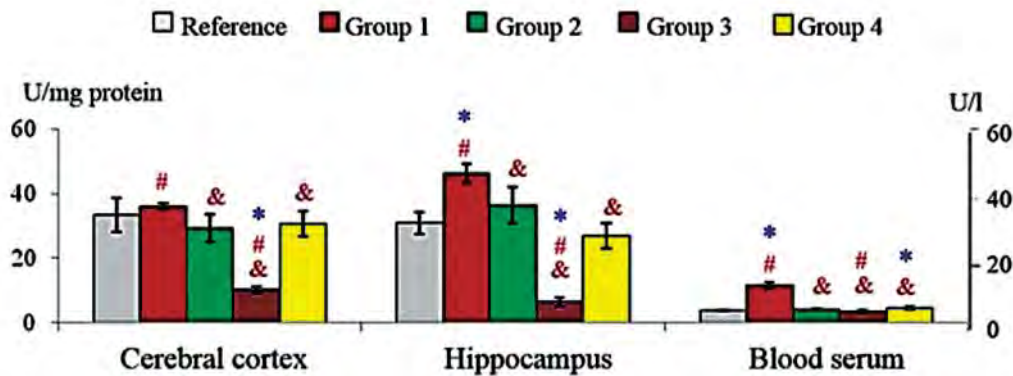


Fig. 1. Effect of 42_Human β -amyloid peptide and curcumin liposome form on angiotensin-converting activity in brain sections (cerebral cortex and hippocampus) and blood serum of the rats

Hereinafter: * — $P \leq 0.05$ compared to control (intact animals, $n = 6$); # — $P \leq 0.05$ when comparing the groups 1 (model of Alzheimer's disease, $n = 6$); 2 (sham operated animals, $n = 6$); 3 (nasal therapy of the AD model animals with liposome curcumin, $n = 6$); 4 (nasal therapy of AD model animals with empty liposomes, $n = 6$), respectively; & — $P \leq 0.05$ compared to the group 1 (Alzheimer's disease model, $n = 6$).

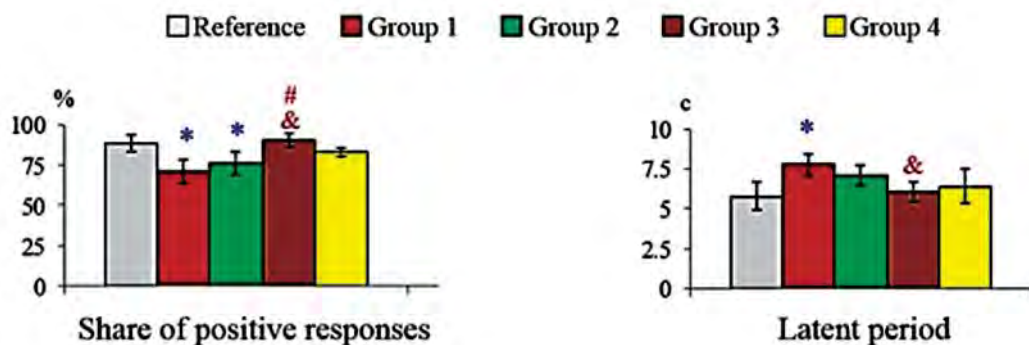


Fig. 2. Dynamics of mnesic ability and memory parameters (share of positive responses and latent period) in effect of 42_Human β -amyloid peptide and curcumin liposome form therapy in the rats

Human in hippocampus of the experimental rats causes neuroinflammation specifically and mainly in the spot of injection. But in the brain cerebral cortex the activation of neuroinflammation was also shown, although to lower extent (Table 2). Namely, only the IL-6 level reliably increased by nearly 54%. Specificity of A β 42_Human inflammatory effect in the brain of the rats was also proved by difference in the levels of the studied cytokines between groups 1 and 2 (Tables 1 and 2).

Effect of liposome curcumin on cytokine levels in hippocampus of the animals was marked by essential suppression of inflammation (Table 1): TNF α level decreased by 56%, IL-6 — by 39% and IL-10 — by 52%, respectively. But not a single cytokine normalized its concentration, unlike in the case with the effect of empty liposomes (Table 1). Effect of CUA as component of liposomes in the cerebral cortex of the

rats with AD gave similar suppression of cytokine response (Table 2): TNF α level decreased by 71%, IL-6 — by 67% and IL-10 — by 41%, respectively. The obtained results are in agreement with our previous data for curcumin water solution only for the brain cerebral cortex of the animals with intrahippocampal injection of A β 42_Human [37]. In hippocampus of the rats with AD model the CUA liposome form showed more intensive suppression of cytokine chain of neuroinflammation compared to its water solution.

The level of peripheral cytokines (TNF α , IL-6 and IL-10 in blood serum) did not reflect neither specific neuroinflammatory effect of 42_Human β -amyloid peptide in the brain of the rats, nor suppression effect of liposome CUA (Table 3). 20–50% rise in TNF α concentration, 54–67% decrease in IL-10 concentration and absence of changes in IL-6 content in blood serum of the rats of all the

Table 1. Effect of curcumin liposome form on TNF α , IL-6 and IL-10 in hippocampus of the rats with the model of Alzheimer's disease

Cytokine	Reference <i>n</i> = 6	Group 1 <i>n</i> = 6	Group 2 <i>n</i> = 6	Group 3 <i>n</i> = 6	Group 4 <i>n</i> = 6
TNF α	50.7 \pm 2.1	63.8 \pm 3.5*#	46.8 \pm 1.9&	28.3 \pm 1.7*#&	46.3 \pm 2.3&
IL-6	57.3 \pm 8.3	72.8 \pm 6.8#	98.3 \pm 6.8*&	44.7 \pm 5.9*&	58.5 \pm 8.2
IL-10	130.4 \pm 11.0	254.3 \pm 16.7*#	152.8 \pm 12.9&	122.4 \pm 13.4*#&	151.0 \pm 12.4&

Note. Hereinafter: the results are represented as $M \pm m$, ng/g of protein.

Table 2. Effect of curcumin liposome form on TNF α , IL-6 and IL-10 in cerebral cerebral cortex of the rats with the model of Alzheimer's disease

Cytokine	Reference <i>n</i> = 6	Group 1 <i>n</i> = 6	Group 2 <i>n</i> = 6	Group 3 <i>n</i> = 6	Group 4 <i>n</i> = 6
TNF α	50.8 \pm 2.5	46.2 \pm 2.4#	40.6 \pm 2.9*&	13.2 \pm 0.9*#&	47.1 \pm 2.8
IL-6	52.5 \pm 4.2	80.8 \pm 7.4*	68.5 \pm 5.8	26.8 \pm 2.0*#&	49.3 \pm 3.9&
IL-10	179.5 \pm 13.0	150.8 \pm 10.6#	206.4 \pm 24.2&	89.4 \pm 7.6*#&	177.0 \pm 12.8&

Table 3. Effect of curcumin liposome form on TNF α , IL-6 and IL-10 in blood serum of the rats with the model of Alzheimer's disease

Cytokine	Reference <i>n</i> = 6	Group 1 <i>n</i> = 6	Group 2 <i>n</i> = 6	Group 3 <i>n</i> = 6	Group 4 <i>n</i> = 6
TNF α	7.9 \pm 0.8	9.5 \pm 0.6 *	10.7 \pm 1.0 *	10.4 \pm 0.4 *#	11.9 \pm 0.9 *&
IL-6	48.3 \pm 10.4	37.5 \pm 6.9	53.2 \pm 11.3	44.8 \pm 7.7	47.0 \pm 14.1
IL-10	19.5 \pm 2.4	9.8 \pm 1.2 *#	6.5 \pm 0.9 *	7.0 \pm 1.8 *#&	9.3 \pm 2.5 *

experimental groups compared to the reference were marked.

The obtained results showed activation of cytokine system in the brain of the rats with AD experimental model (Tables 1, 2). These data are in agreement with other studies on activation of neuroinflammation by A β aggregates [38–41]. A β deposits are responsible for activation of microglia [38]. A β conduces to higher inflammatory response to NF κ B stimulation, the nuclear factor which is involved in regulation of ERK (extracellular signal-regulated kinases) and MAPK (mitogen-activated protein kinases) routes which lead to cytokines and chemokines production [39]. Modification of the inflammatory condition of microglia/macrophages plays prominent role in the course of amyloidosis [40].

Nasal therapy of AD model rats with curcumin liposome form was responsible for suppression of ACE activity and cytokine chain of neuroinflammation. Revealed anti-

inflammatory activity of CUA resulted in recovery of memory parameters and mnesic functions in the animals. Therefore, the previous assumption that its liposome form may be an efficient anti-inflammatory factor in the effect of exogenous β -amyloid peptide was confirmed by experimental data. This natural polyphenol prevents activation of NF κ B transcription nuclear factor suppressing phosphorylation and degradation of I κ B α (NF κ B inhibitor). Since curcumin effect lies in inhibition of IB kinase (IKK) activation, needed for NF κ B activation [42–44], it is just this fact that explains the revealed anti-cytokine effect of curcumin in the experimental animals. The mechanism of curcumin effect on ACE activity is substantiated by the proved suppressor effect of this polyphenol on expression of enzyme gene [45]. Above-mentioned data show high anti-cytokine potential of especially the liposome form of curcumin.

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**ВПЛИВ ЛІПОСОМНОЇ ФОРМИ
КУРКУМІНУ НА АНГІОТЕНЗИН-
ПЕРЕТВОРЮВАЛЬНУ АКТИВНІСТЬ,
ЦИТОКІНИ І КОГНІТИВНІ ВЛАСТИВОСТІ
ЩУРІВ З МОДЕЛЛЮ
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Метою дослідження було вивчення впливу ліпосомної форми куркуміну на активність ангіотензинперетворювального ензиму, цитокині і мнестичні властивості щурів з експериментальною моделлю хвороби Альцгеймера. У тварин з інтрагіпокампульним введенням A β 42_Human застосовували назальну терапію ліпосомною формою куркуміну. Реєстрували концентрацію цитокинів і активність ангіотензинперетворювального ензиму у відділах головного мозку (лобно-фронтальна кора і гіпокамп) та сироватці крові, а також показники умовно-рефлекторної реакції уникнення. У результаті терапії куркуміном встановлено пригнічення активності цитокинів, ангіотензинперетворювального ензиму та відновлення мнестичних показників у щурів із хворобою Альцгеймера. Назальна терапія ліпосомної форми куркуміну мала наслідком зменшення активності ангіотензинперетворювального ензиму та антицитокінового ефекту в цільових відділах головного мозку (лобно-фронтальна кора і гіпокамп), що сприяло відновленню мнестичних властивостей і пам'яті щурів.

Ключові слова: куркумін, ліпосоми, β -амілоїдний пептид, цитокині, ангіотензинперетворювальний ензим.

**ВЛИЯНИЕ ЛИПОСОМНОЙ ФОРМЫ
КУРКУМИНА НА АНГИОТЕНЗИН-
ПРЕВРАЩАЮЩУЮ АКТИВНОСТЬ,
ЦИТОКИНЫ И КОГНИТИВНЫЕ
СВОЙСТВА КРЫС С МОДЕЛЬЮ БОЛЕЗНИ
АЛЬЦГЕЙМЕРА**

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Целью исследования было изучение влияния липосомной формы куркумина на активность ангиотензин-превращающего энзима, цитокины и мнестические свойства крыс с экспериментальной моделью болезни Альцгеймера. У животных с интрагипокампульным введением A β 42_Human применяли назальную терапию липосомной формой куркумина. Регистрировали концентрацию цитокинов и активность ангиотензинпревращающего энзима в отделах головного мозга (лобно-фронтальная кора и гиппокамп) и сыворотке крови, а также показатели условно-рефлекторной реакции избегания. В результате терапии куркумином установлено угнетение активности цитокинов, ангиотензинпревращающего энзима и восстановление мнестических показателей у крыс с болезнью Альцгеймера. Назальная терапия липосомной формой куркумина обусловила уменьшение активности ангиотензинпревращающего энзима и антицитокинового эффекта в целевых отделах головного мозга (лобно-фронтальная кора и гиппокамп), что способствовало восстановлению мнестических свойств и памяти крыс.

Ключевые слова: куркумин, липосоми, β -амилоидный пептид, цитокины, ангиотензинпревращающий энзим.