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Comparative Analysis of Nasal Therapy with Soluble and Liposomal Forms of Curcumin on Rats with Alzheimer's Disease Model

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Abstract

The aim of this study was a comparative analysis of the efficacy of nasal therapy of curcumin soluble and liposomal forms of animals with a model of Alzheimer's disease. Cognitive tests and immunity-enzyme analysis of cytokines were completed. On experimental model of Alzheimer's disease in rats (intrahippocampus administration of 15 nM Aβ42_Human) installed more efficient nasal therapy (1 month treatment) of curcumin liposome form (3.5 μg/animal daily) has turned out more efficient compared to its aqueous solution, both in terms of cognitive tests (the portion of the positive responses and latent period of the conditioned reflex reaction elimination) and in neuroinflammation (cytokines content (interleukin-1β, interleukin-6, interleukin-10, tumor necrosis factor α) in the brain sections: cerebral cortex and hippocampus of the rats with Alzheimer's disease model). The data indicate a high anticytokine potential specifically of the liposomal form of curcumin.

Keywords: Curcumin; Liposome; Alzheimer's disease; Memory; Cytokines; Brain

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease, which is characterised clinically by the progressive loss of short-term memory and cognitive functioning. Major pathological features of an AD brain include the accumulation of extracellular plaques and fibrils with Aβ peptides, intracellular neurofibrillary tangles (NFT), as well as chronic inflammation and widespread synaptic and neuronal loss, leading to brain atrophy and dysfunction [1-3]. With the ageing of many populations worldwide, it is predicted that over the next few decades there will be a marked increase in the number of people with dementia. According to the World Health Organization (WHO), 5% of men and 6% of woman above the age of 60 years are affected with Alzheimer's type dementia worldwide. Current estimations show that 36 million people worldwide have dementia, which is predicted to more than triple to 115 million by 2050 (Figure 1) [4].

The "amyloid cascade hypothesis," in which mutations in amyloid precursor protein, β-secretase (BACE-1), apolipoprotein E, presenilin-1 or presenilin-2 genes lead to increased production of β-amyloid, is now widely considered to contribute to the neurodegeneration seen in AD [5]. Mutations in these genes are linked to some forms of AD [6-8] and although generally responsible for early-onset disease, they have also been reported in some patients with late-onset disease [9,10]. Nevertheless, only about 5% of AD cases are caused by these mutations, and so it seems that there must be other factors that lead to an overproduction and deposition of β-amyloid. The remaining 95% of cases of this neurodegenerative pathology are caused by age-related metabolic disorders: chronic neuroinflammation, oxidative stress, epigenetic alterations and other. Therefore, the main focus of the prevention and treatment of AD may be a correction of nonspecific age-related disorders caused by broad spectrum agents [11,12].

With no current effective disease-modifying treatments available, finding pharmacological/non-pharmacological strategies to halt or slow disease progression is of significant importance. The continuing lack of effective pharmaceutical drugs has also prompted the evaluation of alternative therapeutics, such as nutraceuticals. Extensive studies in the last two decade suggested that curcumin possesses anti-inflammatory, antioxidant, Aβ-lowering agent and Aβ aggregation inhibitor properties; it shows potential as a therapeutic for AD [13-37]. The mechanism for these effects involves modulation of several signaling transduction pathways (Figure 2).

However, curcumin's clinical application is severely limited because of its poor stability under physiological conditions that limits its systemic bioavailability [38,39]. Major reasons contributing to the low plasma and tissue levels of curcumin appear to be due to poor absorption, rapid metabolism, and rapid systemic elimination. Therefore, many technologies have been developed and applied to overcome this limitation and development of nano-sized delivery systems for curcumin, including liposomes, polymeric nanoparticles and micelles, conjugates, peptide carriers, cyclodextrins, solid dispersions, lipid nanoparticles and emulsions [40-46].

Still one problem is physiological barriers (including a blood brain barrier) limiting curcumin absorption after oral or intravenous administrations [47]. This natural polyphenol was more permeable under acidic conditions, but the permeability was substantially below the permeability of highly permeable standards by its non-specific binding. This can be explained by greater stability of keto form of curcumin, which becomes dominant in a tautomeric mixture of keto and enol forms in curcumin's solution. Therefore, the aim of this study was a comparative analysis of the efficacy of nasal therapy of curcumin soluble and liposomal forms of animals with a model of Alzheimer's disease.

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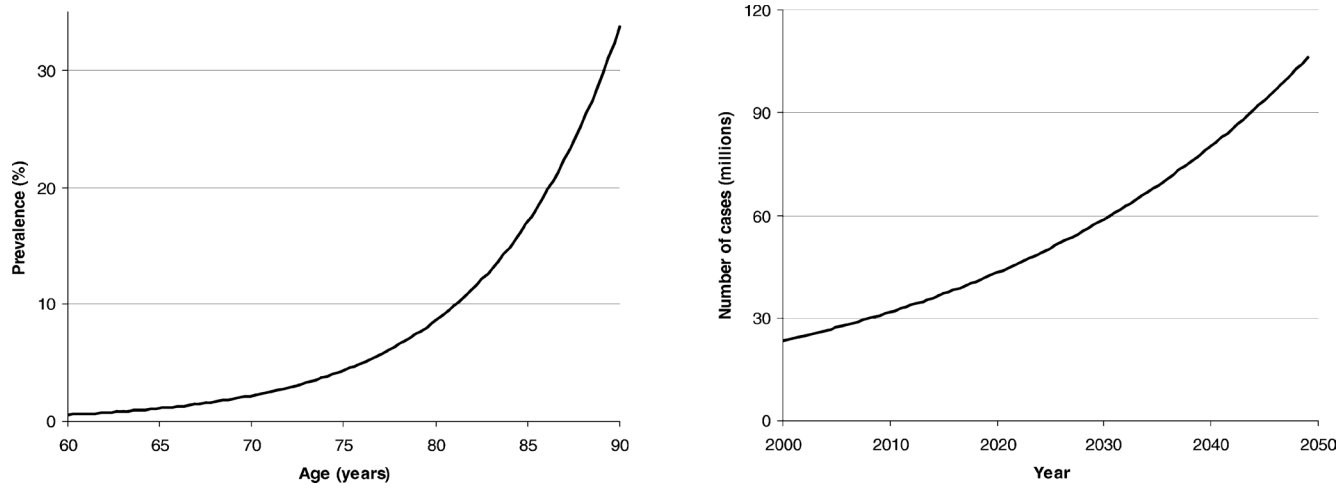

Figure 1: Prevalence of Alzheimer's disease (AD) as a function of age (left) and total number of cases as a function of calendar year (right). Based on data from the Delphi consensus study [4].

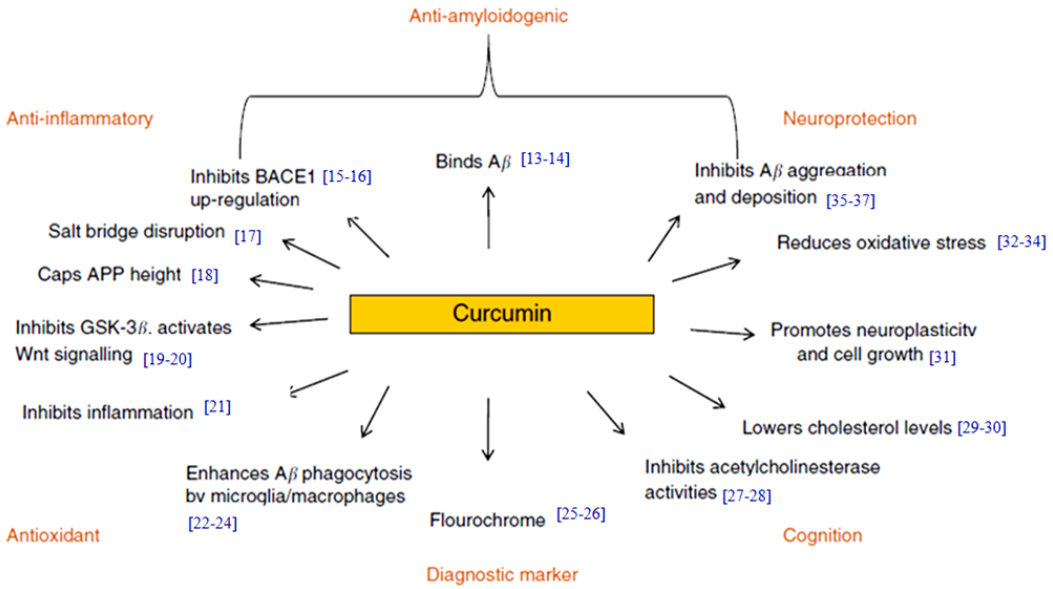

Figure 2: Curcumin: Reported mechanisms of action [1]. The scheme shows the pathways and mechanisms of anti-amyloidogenic, anti-inflammatory, antioxidant, neuroprotective and other effects of curcumin.

Materials and Methods

Study design

The effect of nasal therapy with curcumin in two forms was studied, namely its aqueous solution and its liposomal form (composed of phospholipid/cholesterol liposomes). Experimental protocols were complied with the rules of the European Convention for Protection of Vertebrate Animals used in experiments and for other scientific purposes. The rats (n=60) were randomly distributed into 7 groups. The reference group included the intact animals (n=12); Group 1 included rats 1 month after intrahippocampal injection of Aβ42_Human (Human Amyloid β Protein Fragment 1-42, Sigma-Aldrich, USA) – experimental model of AD (n=12); Group 2 included sham-operated animals (n=12); Group 3 included rats with experimental model of AD, who received curcumin aqueous solution of (Sigma-Aldrich, USA) delivered by intranasal administration daily for 1 month (n=6); Group 4 included the animals with experimental model of AD, who received bidistilled water by intranasal administration daily for 1 month (n=6); Group 5 included rats with experimental model of AD, who received liposomal curcumin delivered by intranasal administration daily for 1 month (n=6) and Group 6 included animals with experimental model of AD, who received intranasal administration of empty liposomes daily for 1 month, as well (n=6).

Cognitive tests

Preliminarily, 20 days before a conditioned reflex reaction was formed in all the rats on the basis of non-conditioned reflex elimination [48]. Infallible conditioned reflex responses to metronome sound were

considered to be positive results. Next to the positive response portion (number) (in percent, %), the duration of latent period of conditioned reflex reaction elimination was registered in the study (in seconds, s). The animals of all the groups were tested for these values of conditioned reflex reaction elimination after the AD experimental model was formed in them and after intranasal administration of curcumin, respectively.

Experimental model of Alzheimer's disease

Aβ42_Human solved in bidistilled water was aggregated for 24 h 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 μg) to the brain hippocampus of the rats. The volume of the solution: 10 μl per animal. Aβ42_Human solution volume was 10 μl, the rate of introduction of a needle syringe chromatographic – 0.03 μl/s and duration of administration – 5 min. The stereotaxic coordinates of the left hippocampus were determined by the map of the rat brain [49], 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.

Nasal therapy with curcumin

Since curcumin has low solubility in water, its concentrated solution in 96% ethanol was first prepared. Curcumin remained stable in ethanol at the room temperature for three weeks but degraded fast in water at neutral or weak basic pH [50]. Therefore the outgoing curcumin solution was dissolved in the bidistilled water to 0.7 g/l immediately before the nasal administration into the rats in the dosage of 3.5 μg/animal.

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 Na_2HPO_4, 1.76 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) was added and thoroughly mixed for suspension of liposomes with curcumin formation [51]. The suspension of empty liposomes was prepared using the similar protocol but at the final stage PBS buffer without CUA was added. Liposome suspension with CUA was 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. Suspension of empty liposomes was diluted similarly. 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 samples of the cerebral cortex and hippocampus were frozen and stored for further measurement. The tissues of the brain sections were homogenized in Tris buffer (50 mM tris-HCl, 150 mM NaCl, pH 7.5), centrifuged at 14,000 g (RS-6, RF) for 5 min and then the supernatant was collected.

Immunity-enzyme analysis of cytokines

The samples of hippocampus supernatant and cerebral cortex were used to determine cytokines by the ELISA method in accordance with the protocols (Rat ELISA Kits Invitrogen BCM DIAGNOSTICS, USA) for IL-1β, IL-6, IL-10 and TNFα. Assay absorption was read out by GBG Stat FAX 2100 (USA) microplate analyzer at 450 nm with wavelength correction at 630 nm. The ELISA data (μg/l cytokins) were recalculated to the general protein. Concentration of general protein was quantitated by Lowry method [52].

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. Values at $p<0.05$ were considered significant.

Results and Discussion

The rats with Alzheimer's disease model (Group 1) showed memory impairment in cognitive tests by such indicators as the portion of the positive responses and latent period of the conditioned reflex reaction elimination (Table 1). In particular, the percentage of positive responses decreased and the length of the latent period increased as compared to the intact animals (Reference group). However, the first indicator also decreased in sham-operated rats (Group 2), calling into question its specificity. In addition, the latter did not show any credible changes in the latent period. Therefore, this particular indicator of animal cognitive tests is a sensitive marker of memory impairment at early stages of dementia. The first clinic symptoms of dement changes manifest themselves primarily in the extension of recollection intervals, and later in the impossibility to recall anything at all.

Daily nasal therapy with aqueous solution of curcumin for 1 month in AD model rats (Group 3) recovered only the portion of the positive responses, whereas latent period remained somewhat extended (Table 1). In the reference group, which received only the solvent, (Group 4) the cognitive indicators under study did not improve. Nasal therapy with liposomal form of curcumin (Table 1) within the same period of time led to a specific recovery of both indicators of the conditioned reflex reaction elimination in animals of Group 5, as compared to Group 1 (AD model animals) and Group 6 (nasal therapy of the AD model rats with empty liposomes). In particular: the portion of the positive responses increased by 19% and 9%, but latent period decreased by 28% and 5%, respectively.

Comparison of the effects of nasal therapy with CUA soluble and

Group	Portion of positive responses (%)	Latent periods (seconds, s)
Reference	88.9 ± 1.2	5.8 ± 0.4
Group 1	76.4 ± 3.3 [1)]	7.8 ± 0.1 [1)]
Group 2	71.0 ± 1.2 [1)]	7.1 ± 0.3
Group 3	87.2 ± 2.0 [2) 3)]	6.7 ± 0.2
Group 4	78.4 ± 3.1 [1)]	7.0 ± 0.2 [1)]
Group 5	91.1 ± 3.7 [2) 3)]	6.1 ± 0.3 [3) 4)]
Group 6	83.4 ± 3.4	6.4 ± 0.4

Remark: [1)]$p \le 0.05$ compared to the Reference (intact animals, n=12); [2)]$p \le 0.05$ at comparison of the Group 1 (Alzheimer's disease model, n=12) and Group 2 (sham-operated animals, n=12), Group 3 (nasal therapy of the AD model rats with soluble CUA, n=6) and Group 4 (nasal therapy of the AD model rats with H_2O, n=6) and Group 5 (nasal therapy of the AD model rats with liposomal CUA, n=6) and Group 6 (nasal therapy of the AD model rats with empty liposomes, n=6), respectively; [3)] $p \le 0.05$ compared to the Group 1 (Alzheimer's disease model, n=12); [4)]$p \le 0.05$ at comparison of the Group 3 (nasal therapy of the AD model rats with soluble CUA, n=6) and Group 5 (nasal therapy of the AD model rats with liposomal CUA, n=6)

Table 1: Effect of curcumin soluble and liposomal forms on memory parameters in the rats with Alzheimer's disease model.

liposomal forms on the memory of animals with a model of Alzheimer's disease, Table 1 shows that the indicators of cognitive tests in Group 5 (nasal therapy of the AD model rats with liposomal CUA) and Group 3 (nasal therapy of the AD model rats with soluble CUA) differ only by 5% and 10% for the portion of the positive responses and latent period, respectively. However, the difference was in favor of liposomal curcumin. Thus, greater efficacy of nasal therapy with liposomal curcumin was established based on the results of cognitive tests.

To determine the leading mechanism of curcumin action at the molecular level, we investigated its impact on the cytokine link of inflammation in targeted parts of the brain of rats (hippocampus and cerebral cortex), which are responsible for the memory. Since brain neurons form a single neuronal network with projections of the neocortex axons in the zones of the hippocampus, the spread of the inflammatory process is expected when the hippocampus is damaged in the cerebral cortex.

Homoaggregates Aβ42_Human in the hippocampus of rats with Alzheimer's disease model caused chronic neuroinflammation specifically and predominantly at the injection site (Table 2). In particular: hippocampal levels IL-1β and IL-10 in rats with Alzheimer's disease model were increased compared with the control group (by 221% and 111%, respectively) and group 2 (110% and 78%, respectively). In this part of the brain, concentration of IL-6 in rats of group 1 did not differ from the values of intact animals (control group), but was reduced by 44% compared to the concentration of IL-6 in the sham-operated rats. However, the activation of the inflammatory process was also found in the cerebral cortex of the rats, although to a lesser extent: increased levels of IL-1β (by 109%) and IL-6 (by 54%) compared with those of intact animals (control), and increased concentrations of IL-6 (by 29%) and reduced IL-10 (by 31%) compared to sham-operated animals. The content of TNFα in the cerebral cortex and hippocampus of rats of groups 1 and 2 did not differ from the benchmark and among themselves (Table 2). This result confirms the conclusion of previous studies [53], in which it was shown that homoaggregates Aβ40_Human, injected into the cerebral cortex of rats, cause greater cytokine response specifically in the area of administration.

Thus, the predictor and catalyst of AD is the chronication of the nonspecific neuroinflammatory process, which provokes the toxicity of Aβ40/42 aggregates. One of the mechanisms of the pathogenesis of amyloidosis is activation of the cytokine response to a local excess of β-amyloid peptides. On the other hand, an excess of proinflammatory cytokines (IL-1β, TNFα, IL-6) against a background of lack of anti-inflammatory interleukins (IL-10) results in an amyloidogenic processing scenario for amyloid precursor protein and new portions of secreted Aβ as a signal peptide of the inflammatory response.

The results obtained with regard to the cytokine system activation in the brain of rats with Alzheimer's disease model are consistent with other studies on the activation of neuroinflammation with Aβ aggregates [54-56]. Aβ deposits are responsible for the activation of microglia [57]. Aβ enhances the inflammatory response to NFκB stimulation, which is involved in the regulation of extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK), leading to the production of cytokines and chemokines [58]. Toll-like receptors (TLR), along with inflammatory cytokine receptors, are important for the regulation of microglial response to Aβ. Modification of microglia inflammatory state plays a leading role in the course of amyloidosis [59]. Generally, neuroinflammation is implicated in the pathogenesis of many neurodegenerative disorders. In AD, several mediators in the inflammation cascade contribute both to neurodegeneration and to the production and accumulation of the β-amyloid peptide, including proinflammation cytokines, phospho-c-Jun NH_2-terminal kinase (pJNK), Wnt signalling, reactive oxygen species, inducible nitric-oxide synthase-mediated production of reactive NO species, and lipid peroxidation products [60].

The effect of an aqueous solution of CUA in cerebral cortex of rats showed specific inhibition of inflammatory cytokine activation (Table 2): normalized levels of IL-1β and IL-6; TNFα levels decreased by 49% compared to control; concentration of IL-10 did not change. In group 4 (nasal administration of solvent - H_2O) we observed further aggravation of the neuroinflammatory process induced by the intrahippocampal administration Aβ42_Human. Concentrations of IL-1β and IL-10 in this part of the brain increased by 50% and 73%, respectively, for one

Group	IL-1β (ng/g protein)	TNFα (ng/g protein)	IL-6 (ng/g protein)	IL-10 (ng/g protein)
Cerebral cortex				
Reference	166.3 ± 18.5	50.8 ± 2.5	52.5 ± 4.2	179.5 ± 13.0
Group 1	347.5 ± 11.8 [1)]	46.2 ± 2.4 [2)]	80.8 ± 7.4 [1)]	150.8 ± 10.6 [2)]
Group 2	340.8 ± 13.3 [1)]	40.6 ± 2.9 [1) 3)]	68.5 ± 5.8	206.4 ± 24.2 [3)]
Group 3	222.0 ± 16.1 [1) 2) 3)]	26.1 ± 3.7 [1) 2) 3)]	45.2 ± 5.7 [2) 3)]	133.6 ± 10.2 [1) 2)]
Group 4	430.5 ± 20.6 [1) 3)]	58.6 ± 2.5 [3)]	79.1 ± 5.6 [1)]	281.3 ± 8.3 [1) 3)]
Group 5	-	13.2± 0.9 [1) 2) 3) 4)]	26.8 ± 2.0 [1) 2) 3) 4)]	89.4 ± 7.6 [1) 2) 3) 4)]
Group 6	-	47.1 ± 2.8	49.3 ± 3.9 [3)]	177.0 ± 12.8 [3)]
Hippocampus				
Reference	174.0 ± 18.8	50.7 ± 2.1	57.3 ± 8.3	130.4 ± 11.0
Group 1	558.0 ± 18.8 [1) 2)]	63.8 ± 3.5 [1) 2)]	72.8 ± 6.8 [1) 2)]	254.3 ± 16.7 [1) 2)]
Group 2	365.4 ± 19.1 [1) 3)]	46.8 ± 1.9 [3)]	98.3 ± 6.8 [1) 3)]	152.8 ± 12.9 [3)]
Group 3	523.6 ± 14.8 [1) 2)]	68.0 ± 4.3 [1) 2)]	101.1 ± 7.1 [1) 2) 3)]	362.0 ± 21.1 [1) 2) 3)]
Group 4	581.3 ± 16.9 [1)]	80.0 ± 7.5 [1) 3)]	120.6 ± 11.8 [1) 3)]	505.3 ± 20.8 [1) 3)]
Group 5	-	28.3 ± 1.7 [1) 2) 3) 4)]	44.7 ± 5.9 [3) 4)]	122.4 ± 13.4 [2) 3) 4)]
Group 6	-	46.3 ± 2.3 [3)]	58.5 ± 8.2 [3)]	151.0 ± 12.4 [3)]

Remark: [1)]p ≤ 0.05 compared to the Reference (intact animals, n=12); [2)]p ≤ 0.05 at comparison of the Group 1 (Alzheimer's disease model, n=12) and Group 2 (sham-operated animals, n=12), Group 3 (nasal therapy of the AD model rats with soluble CUA, n=6) and Group 4 (nasal therapy of the AD model rats with H_2O, n=6) and Group 5 (nasal therapy of the AD model rats with liposomal CUA, n=6) and Group 6 (nasal therapy of the AD model rats with empty liposomes, n=6), respectively; [3)]p ≤ 0.05 compared to the Group 1 (Alzheimer's disease model, n=12); [4)]p ≤ 0.05 at comparison of the Group 3 (nasal therapy of the AD model rats with soluble CUA, n=6) and Group 5 (nasal therapy of the AD model rats with liposomal CUA, n=6)

Table 2: Effect of curcumin soluble and liposomal forms on cytokines content in the brain sections of the rats with Alzheimer's disease model.

month of bidistillate nasal therapy. In the hippocampus of animals, the impact of curcumin on cytokine indicators had a similar orientation (Table 2). However, the concentration of any of the cytokines did not normalize, while the levels of IL-6 (49%) and IL-10 (83%) increased compared with the figures earlier this month. But a comparison of cytokines in hippocampus of rats in groups 3 and 4 clearly indicates a specific reduction in levels of IL-1β (by 33%), TNFα (by 24%), IL-6 (34%) and IL-10 (by 99%) due to a depressing effect of curcumin. The anticytokine effect of CUA can be explained by its ability to inhibit the activation of the inflammatory transcription factor NFκB, inhibiting phosphorylation and degradation of IκBα (NFκB inhibitor) [61,62]. Recently discovered a network of routes anti-inflammatory impact of curcumin in conditions of neurotoxicity units of β-amyloid peptide: inhibit cyclooxygenase (COX-2), phospholipases, pJNK, transcription factor AP-1 and NF-κB [63-65], which makes its properties as a potent inhibitor of pro-inflammatory cytokine production [66].

The effect of liposomal curcumin on the cytokines performance in the hippocampus of animals after the intrahippocampal administration of Aβ42_Human was marked with a significant inhibition of inflammation (Table 2): TNFα levels decreased by 56%, IL-6 – by 39% and IL-10 – by 52%, respectively. However, the concentration of cytokines did not normalize. The effect of CUA in the composition of liposomes in cerebral cortex of rats with Alzheimer's disease model showed similar inhibition of cytokine reactions: TNFα levels decreased by 71%, IL-6 – by 67% and IL-10 – by 41%, respectively.

Conclusion

The results obtained coincide with the data on the aqueous solution of curcumin only for cerebral cortex, because the liposomal form of CUA in the hippocampus of rats with Alzheimer's disease model showed a more intense suppression of cytokine level of neuroinflammation compared to its aqueous solution. The above data indicate a high anticytokine potential specifically of the liposomal form of curcumin.

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