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EFFECT OF CURCUMIN ON ACCUMULATION IN MONONUCLEAR CELLS AND SECRETION IN INCUBATION MEDIUM OF $A\beta_{40}$ AND CYTOKINES UNDER LOCAL EXCESS OF $A\beta_{42}$-HOMOAGGREGATES

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The aim of the work was to investigate accumulation of endogenous $A\beta_{40}$ and cytokines (IL-1β, TNFα, IL-6, IL-10) in mononuclear cells and their secretion into incubation medium under $A\beta_{42}$-aggregates' toxicity and anti-inflammatory effects of curcumin. Mononuclear cells were isolated in Ficoll-Urografin density gradient from venous blood of healthy donors, resuspended and used for testing of homoaggregates of $A\beta_{42}$ (15 nM), curcumin (54 pM) and their combinations on various timescales (0, 1, 2, 3, 6 and 24 hours). Endogenous $A\beta_{40}$ and cytokines were detected in mononuclear cells and (separately) in incubation medium by ELISA. We demonstrated for the first time that homoaggregates of $A\beta_{42}$ cause rapid accumulation of endogenous $A\beta_{40}$ in mononuclear cells and accelerate its secretion into incubation medium. We found increased concentration of TNFα after 3 hours of incubation, and no changes in IL-1β concentration due to secretion of these pro-inflammatory factors into incubation medium. The concentrations of IL-6 in mononuclear cells were increased under effects of $A\beta_{42}$ homoaggregates, and it was being secreted profoundly into incubation medium. $A\beta_{42}$ did not affect IL-10 secretion, yet caused an increase in its intracellular concentration after 1 hour of incubation, which was subsequently suppressed. Curcumin prevented the increase in $A\beta_{40}$ concentration in mononuclear cells and significantly decreased its secretion resulting from $A\beta_{42}$ toxicity. Curcumin negated the activating effect of $A\beta_{42}$ on pro-inflammatory cytokines, starting immediately for IL-1β and on 3-6 hours for TNFα, which resulted in decreased extracellular concentrations of these cytokines. The polyphenol also potentiated replenishing of intracellular IL-6 and IL-10 concentrations and their secretion into incubation medium.

K e y w o r d s: curcumin, β-amyloid peptides 40 and 42 ($A\beta_{40}$, $A\beta_{42}$), cytokines, secretion, human peripheral blood mononuclear cells.

Alzheimer's disease is the primary cause of senile dementia associated with amyloid-β peptides (Aβ), oligomers and aggregates of which exert toxic and destructive effects upon neural tissue [1-5]. Nevertheless, there is no clear evidence of the agent that provokes increased synthesis of amyloid-β precursor protein (AβPP) and the switch in its processing towards amyloid pathway followed by local accumulation of Aβ. Aβ is generally considered to be a toxic molecular waste product [6-8]. Yet recent studies have demonstrated trophic [9] properties of AβPP (antimicrobial, in particular), that served as basis for its comparison with other antimicrobial peptides (defensins, histatins, and cathelicidins) and attribution to brain's innate immune system [10]. We have proved [11] the role of chronic inflammation in initiation of amyloidosis, and the involvement of cytokines and Aβ in inflammatory response to toxic effects of Aβ-aggregates.

The variable dynamics of Aβ ($A\beta_{40}$ and $A\beta_{42}$) in blood serum and cerebrospinal fluid of patients with amyloidosis has been demonstrated [12, 13]. The Aβ-levels in peripheral blood flow were highly increased during initial asymptomatic stages of amyloid-associated pathology, on the other hand $A\beta_{40}$ and $A\beta_{42}$ levels in patients during neurodegenerative stages of the Alzheimer's disease were within or even below normal margins [14, 15]. The presence of these neuropeptides in biological fluids was attributed to Aβ accumulation in certain parts of brain (hippocampus, frontal cortex, and olfactory bulbs) and to increased permeability of blood-brain barrier due to inflammation [16, 17]. In contrast, we have established [18] that AβPP expression and its

amyloidogenic processing in human peripheral blood mononuclear cells is activated in response to $A\beta_{42}$ effects and leads to $A\beta_{40}$ accumulation regardless of neural tissue. One of the aims of the present work was to establish the possibility of secretion of $A\beta_{40}$ produced by peripheral mononuclear cells into surrounding medium, which could be used as its indicator in biological fluids of patients with Alzheimer disease and of its function other than being an amyloidohenesis.

Another standing problem is finding approaches to eliminate causes for AβPP overexpression and amyloidogenic processing, and inhibiting the inflammation resulting from Aβ-aggregate toxicity. We have demonstrated curcumin efficiency as a regulator of cytokine-dependent inflammation *in vivo* and *in vitro* [18-20]. Therefore it was sensible to investigate the effect of curcumin on accumulation and secretion of endogenous $A\beta_{40}$ and cytokines by mononuclear cells of human peripheral blood in vitro under $A\beta_{42}$ aggregates' toxicity.

Materials and Methods

The experiments were conducted in accordance with provisions of the Universal Declaration on Bioethics and Human Rights (UNESCO, 2005).

Peripheral blood mononuclear cells were isolated ex tempore in Ficoll-Urografin density gradient from venous blood samples of three healthy donors (separately). The cells were washed thrice with sterile normal saline at room temperature and resuspended in RPMI medium in aliquots of 2×10^6 cells/ml. The resulting samples (n = 3) were subjected to $A\beta_{42}$ (15 nM), curcumin (54 pM) and their combination (with the same concentrations) at various timeframes. The ratio of volumes of effectors to cell suspension was 1:100.

$A\beta_{42}$_Human (Human Amyloid β Protein Fragment 1-42, Sigma-Aldrich, USA) was dissolved in double-distilled water and aggregated for 24 h at 37 °C. Large crude $A\beta_{42}$ agglomerates were disintegrated by ultrasound and sterilized prior to application.

As curcumin is water-insoluble, the primary solution was first dissolved in 96% ethanol and then diluted to 0.7 g/l immediately prior to addition to cell suspension.

The effect of $A\beta_{42}$ and 0.9% NaCl on mononuclear cells was investigated in 0-, 1-, 2-, 3-, 6-, and 24-hour incubation experiments at 37 °C and 600 rpm mix. The effects of curcumin alone and curcumin combined with $A\beta_{42}$ were investigated in 2-, 3-, 6-, and 24-hour experiments under the same conditions; curcumin was added after 1 hour incubation with $A\beta_{42}$ or normal saline (0.9% NaCl). Cells were sampled at the mentioned time points (2×10^6 cells/ml), sedimented by centrifugation and disintegrated by ultrasound (MUSSON-1 ultrasound inhalator, 3 min treatment at 2.64 MHz wavelength and 0.25 W/cm^3 intensity) the samples were then centrifuged at 6000 rpm for 20 min. Cell supernatant and incubation medium aliquots were used for the enzyme-linked immunosorbent assay (ELISA).

Concentrations of endogenous $A\beta_{40}$ and cytokines were estimated by ELISA in accordance with kit manuals (Vektor-Best, Russia) for IL-1β, IL-6, IL-10, and TNFα, and *ELISA Kit Human* $A\beta_{40}$ (Invitrogen, USA). Absorptions were measured with GBG STAT-FAX 2100 (USA) at 450 nm and correction at 630 nm. The results were calculated against total protein concentration (ng/g of protein) measured by Lowry method [21].

Mean values and standard deviations were determined for the indicators of mononuclear cells suspension. The statistical analysis was performed with Student's *t*-test, the differences were considered significant at $P < 0.05$.

Results and Discussion

Study of $A\beta_{42}$ and curcumin's effects on inflammation dynamics in suspension of mononuclear cells include *in vitro* determination of intracellular accumulation of $A\beta_{40}$ and cytokines (IL-1β, TNFα, IL-6, and IL-10) and their secretion into incubation medium. The results (Fig. 1) demonstrate a 2-fold increase in endogenous $A\beta_{40}$ concentration under incubation of mononuclear cells with normal saline or curcumin for 6 h, which is non-specific and may be explained by a spontaneous flux in expression or processing of AβPP [18]. $A\beta_{42}$ homoaggregates caused 7.7-fold increase in $A\beta_{40}$ concentration in mononuclear cells and activation of its secretion on the 1st h of incubation. The described [18] early and rapid activation of AβPP processing in mononuclear cells under $A\beta_{42}$ toxicity led to notable increase of intracellular $A\beta_{40}$ levels as well as to forced secretion of this mediator of inflammation into incubation medium.

The intracellular $A\beta_{40}$ concentration then gradually decreased for 6-24 hours, but did not recede to the starting levels (0 hour) or the levels in mononuclear cells incubated with normal saline for

24 h. Beginning at 6th h of incubation with $A\beta_{42}$, $A\beta_{40}$ secretion was increased 2-fold and remained elevated afterwards (Fig. 1).

Curcumin had no effect upon $A\beta_{40}$ concentration in suspension of mononuclear cells in comparison with the dynamics displayed by cells incubated with normal saline. Nevertheless, it noticeably inhibited the increase in $A\beta_{40}$ intracellular concentration and secretion caused by $A\beta_{42}$ (Fig. 1). The inhibiting effect of curcumin upon $A\beta_{40}$ production is due to its ability to downregulate GSK-3β mediated activation of presenilin-1 (PS-1) [22]. PS-1 participates in γ-secretase enzyme complex, which takes part in AβPP processing, and is a GSK-3β substrate. The latter modulates γ-secretase activity through phosphorylation of serine in PS-1 loop domain [23]. Curcumin has been demonstrated to increase the proportion of inactivated (Ser9-phosphorylated) GSK-3β form depending on concentration and duration of exposure, and also to inhibit expression of *PS1* and *GSK-3β* genes [22]. These factors lead to diminished Aβ production. Our data are in accordance with the results by others who investigate curcumin effects on the model of Aβ-induced inflammation of primary astrocytes [24] and mouse cortical neurons culture [25].

Thus, $A\beta_{42}$ homoaggregates induced accumulation of endogenous $A\beta_{40}$ in mononuclear cells and stimulated secretion of this pro-inflammatory factor into incubation medium. Curcumin served to substantially prevent the $A\beta_{40}$ cellular concentration increase and significantly decreased its secretion associated with toxic effects of exogenous $A\beta_{42}$.

We observed a noticeable increase in TNFα concentration in mononuclear cells and its secretion under effect of mere normal saline, with the maximum effect on 6th h of exposure (Fig. 2). Unlike the TNFα dynamics, the levels of IL-1β under effects of normal saline fluctuated somewhat close to the baseline. The significant decrease was measured on the 1st and 24th h in cells, and the secretion was registered beginning with the 3rd h of exposure (Fig. 3). We attribute these fluctuations in levels of cytokines to spontaneous activation of mononuclear cells due to isolation-associated stress.

Mononuclear cells are known to principally bear the precursors of pro-inflammatory cytokines (pro-IL-1β and pro-TNFα). The active form of IL-1β is produced by caspase 1 [26], and the active form of TNFα by tumor necrosis factor-alpha converting enzyme (TACE), and the products are rapidly secreted into extracellular matrix. TACE also cleaves AβPP within Aβ membrane domain, thus inhibiting production of β-amyloid peptides [28]. The extracellular IL-1β that had not bound to its receptors (IL1R1 and IL1R2) is degraded by matrix metalloproteinases (MMPs): MMP-1, MMP-2, MMP-3, and MMP-9 [29]. The secreted TNFα binds to the corresponding receptors (TNFR1 and TNFR2) [30].

We noted an increase of TNFα concentration in mononuclear cells (beginning with the 3rd h of exposure) and incubation medium (beginning with the

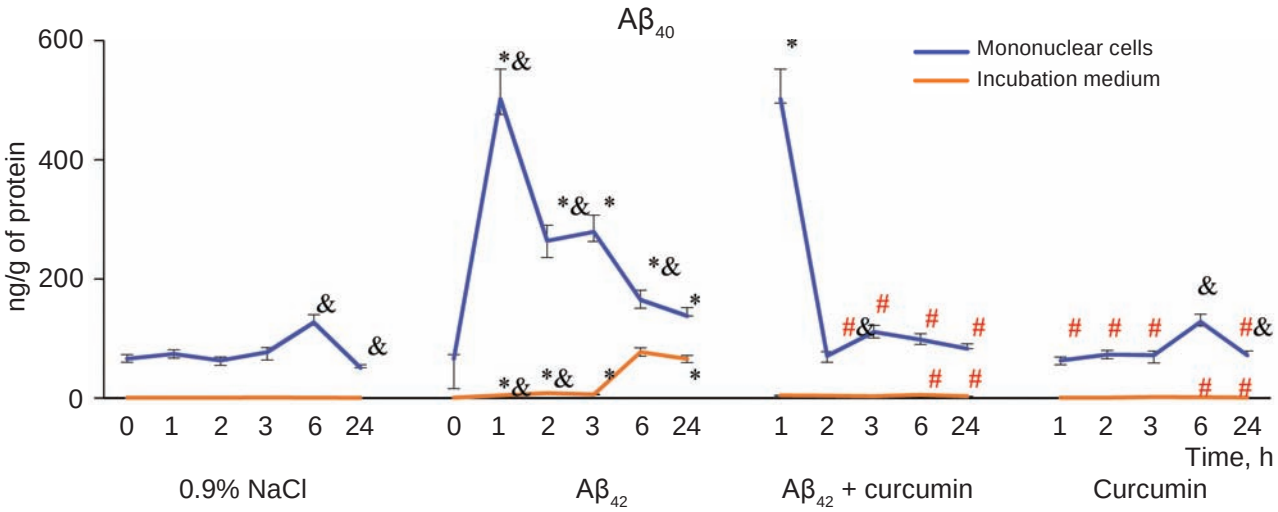

*Fig. 1. $A\beta_{40}$ content in mononuclear cells and incubation medium under effect of normal saline, $A\beta_{42}$, curcumin, and their combination. * Denotes changes with $P < 0.05$ in comparison to normal saline effect; # denotes changes with $P < 0.05$ in comparison to $A\beta_{42}$ effect; & denotes changes with $P < 0.05$ in comparison to the preceding time point*

6th h of exposure) as a specific response to the effects of $A\beta_{42}$ homoaggregates (Fig. 2). Curcumin alleviated this effect of $A\beta_{42}$ within 3 to 6 hours. TNFα levels in mononuclear cells exposed to curcumin alone resembled those of normal saline-exposed cells. These results confirm that curcumin inhibits only the $A\beta_{42}$-induced accumulation of TNFα, and does not affect its spontaneous production.

Curcumin effect on IL-1β concentration after 1-h incubation with $A\beta_{42}$ was apparent on the 1st h of the polyphenol's effect (2 h, Fig. 3). Its total concentration was 33% lower. During subsequent incubation of mononuclear cells with $A\beta_{42}$ and curcumin the IL-1β levels did not differ from the basic ones (Fig. 3), which is probably due to the fact that curcumin has no effect on caspase 1 [31].

We observed increased secretion of the pro-inflammatory cytokines in incubation medium for the duration of incubation (up to 24 h) beginning from the 1st h of exposure for IL-1β and from the 3rd h for TNFα (Fig. 2 and 3) in response to all the effectors and their combination.

Thus, *in vitro* incubation of mononuclear cells with exogenous $A\beta_{42}$ leads to elevated intracellular concentration of TNFα (on the 3rd h of incubation), but not to accumulation of IL-1β in cells, which served to potentiate the release of these cytokines into incubation medium. Curcumin addition alleviated this effect of $A\beta_{42}$ upon cellular concentrations of the pro-inflammatory cytokines, beginning immediately for Il-1β and from 3 to 6 hours for TNFα, which led to their diminished extracellular concentrations.

The dynamics of IL-6 content in mononuclear cells incubated with normal saline (Fig. 4) generally follows that of TNFα, with the exception that intracellular IL-6 level dropped 3.6-fold immediately and then gradually increased towards starting values for 6-24 h, while TNFα content in mononuclear cells increased twofold from the 1st h of incubation and was 4.8 times higher than the starting value after 6 hours. IL-6 secretion into incubation medium was detected at 6-24 h (Fig 4), which differs substantially from the rapid excretion of the pro-inflammatory cytokines of the initial wave of cytokine system: TNFα (beginning with the 1st h) and IL-1β (beginning with the 3rd h).

Exposure to $A\beta_{42}$ homoaggregates, curcumin, or both caused immediate decrease in intracellular concentrations of IL-6 followed by restoration on the 3rd h of incubation. Curcumin subsequently caused gradual increase in accumulation and secretion of the cytokine, and $A\beta_{42}$ homoaggregates caused its decreased accumulation and increased secretion (Fig. 4). Therefore, we established increase in intracellular levels of IL-6 and activation of its secretion into incubation medium under influence of all the tested effectors. The effect of curcumin was substantial on the 24th h of exposure. These data are not in discrepancy with the evidence of the inhibitive

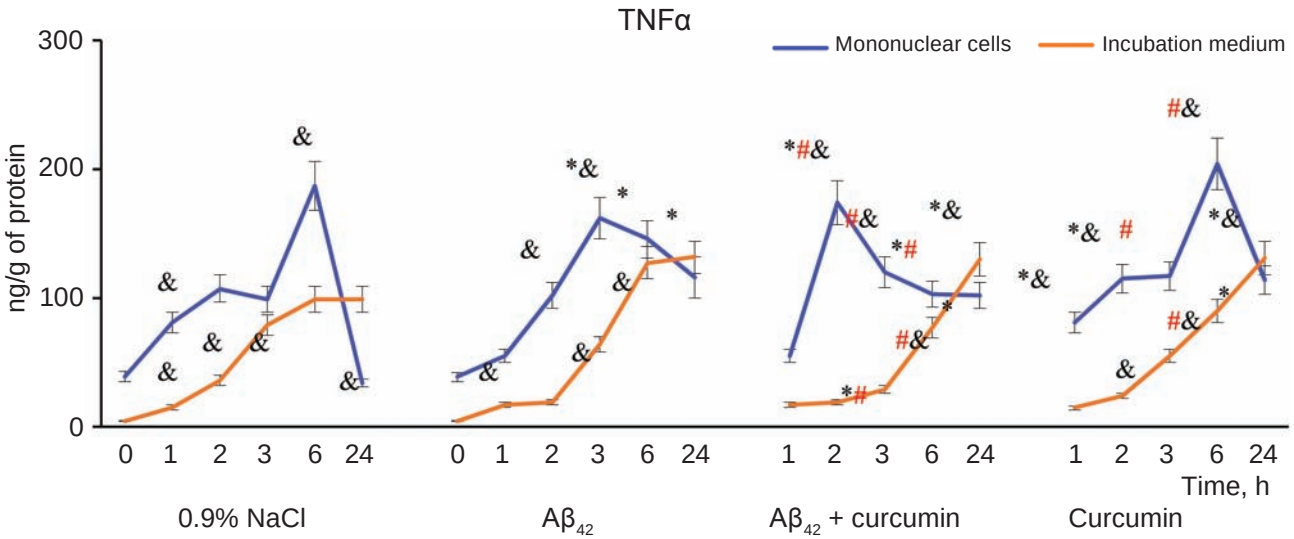

Fig. 2. TNFα content in mononuclear cells and incubation medium under effect of normal saline, $A\beta_{42}$, curcumin, and their combination. Denotes changes with $P < 0.05$ in comparison to normal saline effect; # denotes changes with $P < 0.05$ in comparison to $A\beta_{42}$ effect; & denotes changes with $P < 0.05$ in comparison to the preceding time point*

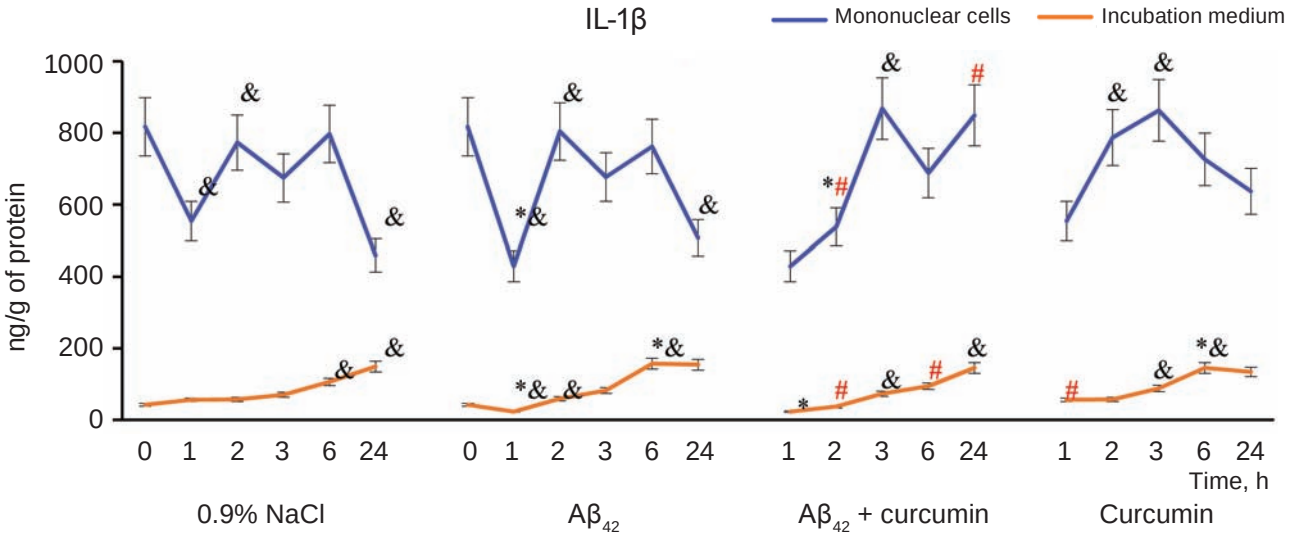

*Fig. 3. IL-1β content in mononuclear cells and incubation medium under effect of normal saline, $A\beta_{42}$, curcumin, and their combination. * Denotes changes with P < 0.05 in comparison to normal saline effect; # denotes changes with P < 0.05 in comparison to $A\beta_{42}$ effect; & denotes changes with P < 0.05 in comparison to the preceding time point*

effects of curcumin on the activation of pro-inflammatory cytokines [32, 33].

$A\beta_{42}$ did not affect IL-10 secretion, yet caused increase in its intracellular concentration after 1 h exposure, with subsequent inhibition of accumulation in cells (Fig. 5). Curcumin addition after 1-h exposure to $A\beta_{42}$ homoaggregates restored intracellular levels of the anti-inflammatory interleukin on 6-24 h of incubation *in vitro*. Curcumin alone caused gradual elevation in intracellular IL-10 content (1-3 h) with increased excretion in incubation medium on 6-24 h (Fig. 5).

These data corroborate our previous results indicating that IL-10 expression is not induced in mononuclear cells under effect of exogenous $A\beta_{42}$, and that concentration of iRNA of IL-10 is increased under effect of curcumin [18]. Others have proven the positive effects of curcumin upon IL-10 expres-

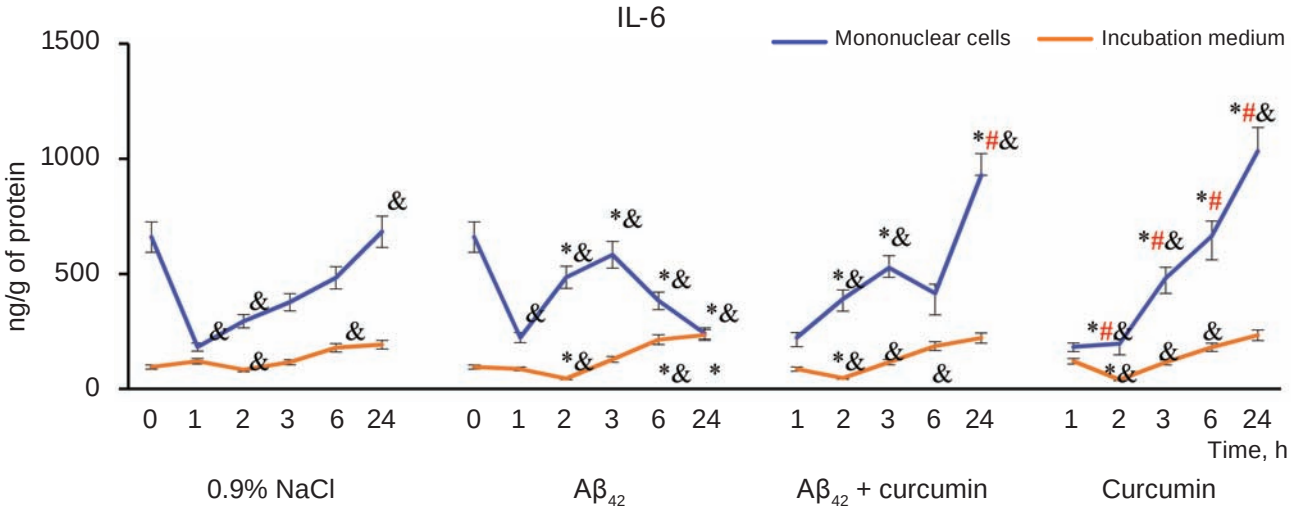

*Fig. 4. IL-6 content in mononuclear cells and incubation medium under effect of normal saline, $A\beta_{42}$, curcumin, and their combination. * Denotes changes with P < 0.05 in comparison to normal saline effect; # denotes changes with P < 0.05 in comparison to $A\beta_{42}$ effect; & denotes changes with P < 0.05 in comparison to the preceding time point*

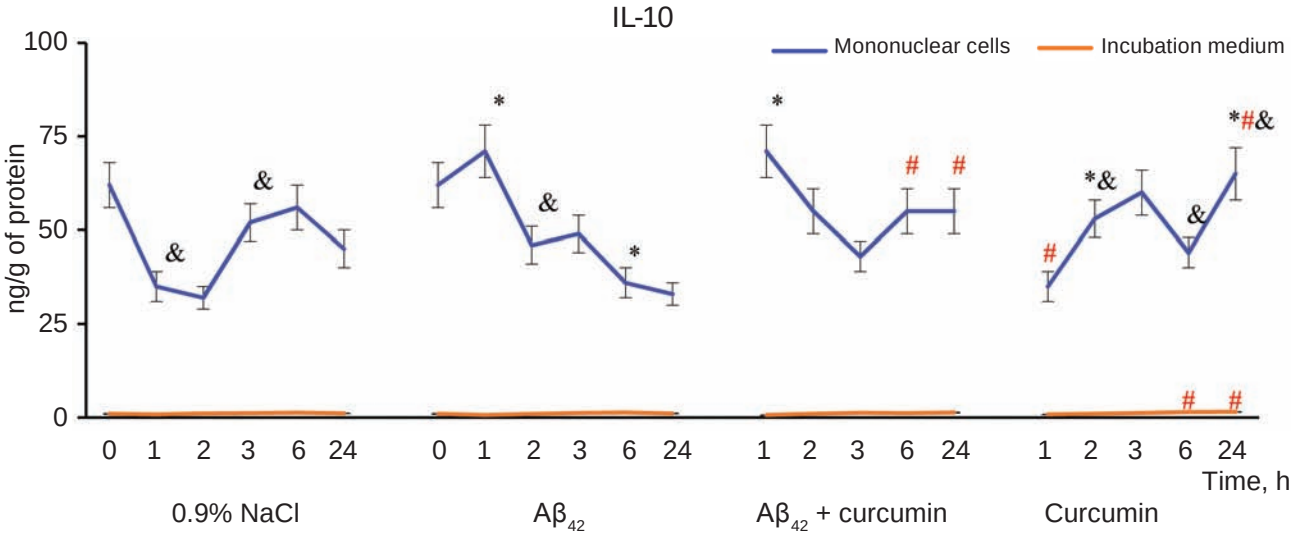

*Fig. 5. IL-10 content in mononuclear cells and incubation medium under effect of normal saline, $A\beta_{42}$, curcumin, and their combination. * Denotes changes with $P < 0.05$ in comparison to normal saline effect; # denotes changes with $P < 0.05$ in comparison to $A\beta_{42}$ effect; & denotes changes with $P < 0.05$ in comparison to the preceding time point*

sion [34, 35], and attributed these *in vivo* effects to inhibition of p38 activity through suppression of its phosphorylation.

Therefore, we hereby establish the capability of mononuclear cells to produce and secrete endogenous $A\beta_{40}$, which indicates its peripheral origin if detected in blood flow. We also demonstrate the non-amyloidogenic function of $A\beta_{40}$ as a pro-inflammatory messenger responsive to effects of $A\beta_{42}$ homoaggregates. We observed for the first time that curcumin prevented increase in $A\beta_{40}$ intracellular concentrations and significantly decreased its secretion under effects of exogenous $A\beta_{42}$. We show the particularities in dynamics of accumulation and secretion by mononuclear cells of the investigated cytokines (IL-1β, TNFα, IL-6, and IL-10) under toxic effects of $A\beta_{42}$ aggregates and anti-inflammatory influence of curcumin.

ВПЛИВ КУРКУМІНУ НА НАКОПИЧЕННЯ В МОНОНУКЛЕАРАХ І НА СЕКРЕЦІЮ В ІНКУБАЦІЙНЕ СЕРЕДОВИЩЕ $Aβ_{40}$ ТА ЦИТОКІНІВ В УМОВАХ ЛОКАЛЬНОГО НАДЛИШКУ ГОМОАГРЕГАТІВ $Aβ_{42}$

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Метою дослідження було вивчення накопичення в мононуклеарах і секреції до інкубаційного середовища ендогенного $Aβ_{40}$ і цитокінів (IL-1β, TNFα, IL-6, IL-10) в умовах токсичного впливу $Aβ_{42}$-агрегатів та антизапального ефекту куркуміну. Суспензію мононуклеарних клітин, ізольованих за допомогою фіколурографінового градієнта зі зразків венозної крові здорових добровольців, використовували для дослідження впливу гомоагрегатів $Aβ_{42}$ (15 нМ), куркуміну (54 пМ) та їх поєднаній дії в динаміці часу (0, 1, 2, 3, 6 і 24 год). Методом імуноензимного аналізу вимірювали вміст ендогенного $Aβ_{40}$ і цитокінів окремо в мононуклеарах і в інкубаційному середовищі. Вперше показано, що гомоагрегати $Aβ_{42}$ обумовлюють швидке накопичення ендогенного $Aβ_{40}$ в мононуклеарах та прискорюють його секрецію до інкубаційного середовища. Встановлено збільшення концентрації TNFα (через 3 год інкубації) і відсутність накопичення IL-1β в клітинах завдяки істотній секреції цих запальних месенджерів до інкубаційного середовища. Виявлено збільшення мононуклеарного пулу IL-6 і активацію його секреції до інкубаційного середовища за дії $Aβ_{42}$-гомоагрегатів. $Aβ_{42}$ не впливав на секрецію IL-10, але обумовлював збільшення його внутрішньоклітинної концентрації через 1 год інкубації з наступним пригніченням клітинного накопичення. Додавання куркуміну запобігало збільшенню концентрації $Aβ_{40}$ в мононуклеарах та вірогідно зменшувало його секрецію, обумовлену токсичним впливом екзогенного $Aβ_{42}$ на клітини. Куркумін знімав активуючий ефект $Aβ_{42}$ на мононуклеарний пул запальних цитокінів: з перших годин дії для IL-1β та в часовому інтервалі 3–6 год для TNFα, що позначилося зменшенням позаклітинного пулу обох цитокінів. Цей поліфенол також сприяв відновленню внутрішньоклітинного пулу IL-6 і IL-10 та позитивно впливав на їх секрецію до інкубаційного середовища.

Ключові слова: куркумін, β-амілоїдні пептиди – 40 і 42 ($Aβ_{40}$, $Aβ_{42}$), цитокіни, секреція, мононуклеари периферійної крові людини.

ВЛИЯНИЕ КУРКУМИНА НА НАКОПЛЕНИЕ В МОНОНУКЛЕАРАХ И НА СЕКРЕЦИЮ В ИНКУБАЦИОННУЮ СРЕДУ $Aβ_{40}$ И ЦИТОКИНОВ В УСЛОВИЯХ ЛОКАЛЬНОГО ИЗБЫТКА ГОМОАГРЕГАТОВ $Aβ_{42}$

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Целью исследования было изучение накопления в мононуклеарах и секреции из них в инкубационную среду эндогенного $Aβ_{40}$ и цитокинов (IL-1β, TNFα, IL-6, IL-10) в условиях токсического действия $Aβ_{42}$-агрегатов и антивоспалительного эффекта куркумина. Суспензию мононуклеарных клеток, изолированных с помощью фиколл-урографинового градиента из образцов венозной крови здоровых добровольцев, использовали для исследования влияния гомоагрегатов $Aβ_{42}$ (15 нМ), куркумина (54 пМ) и их сочетанного действия во временной динамике (0, 1, 2, 3, 6 и 24 ч). Методом иммуноэнзимного анализа измеряли содержание эндогенного $Aβ_{40}$ и цитокинов отдельно в мононуклеарах крови человека и в инкубационной среде. Впервые показано, что гомоагрегаты $Aβ_{42}$ обусловливают быстрое накопление эндогенного $Aβ_{40}$ в мононуклеарах и ускоряют его секрецию в инкубационную среду. Установлено увеличение внутриклеточной концентрации TNFα (через 3 ч инкубации) и отсутствие накопления IL-1β в клетках вследствие существенной секреции этих воспалительных мессенджеров в инкубационную среду. Показано увеличение мононуклеарного пула IL-6 и активация его секреции в инкубацинную среду под действием

$A\beta_{42}$-гомоагрегатов. $A\beta_{42}$ не влиял на секрецию IL-10, хотя и обусловливал увеличение его внутриклеточной концентрации через 1 ч инкубации с последующим угнетением накопления в клетках. Добавление куркумина предотвращало увеличение концентрации $A\beta_{40}$ в клетках и достоверно уменьшало его секрецию, обусловленную токсическим действием экзогенного $A\beta_{42}$. Куркумин снимал активирующий эффект $A\beta_{42}$ на мононуклеарный пул воспалительных цитокинов: с первых часов действия для IL-1β и в интервале времени 3–6 ч для TNFα, что отразилось на уменьшении внеклеточного пула обоих цитокинов. Этот полифенол также способствовал восстановлению внутриклеточного пула IL-6 и IL-10 и положительно влиял на их секрецию в инкубационную среду.

Ключевые слова: куркумин, β-амилоидные пептиды 40 и 42 ($A\beta_{40}$ и $A\beta_{42}$), цитокины, секреция, мононуклеары периферической крови человека.

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