Research Article

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Expression of proteins linked to Alzheimer's disease in C6 rat glioma cells under the action of lipopolysaccharide (LPS), nimesulide, resveratrol and citalopram

[LPS, nimesulid, resveratrol ve sitalopram etkisi altında C6 sıçan glioma hücrelerinde Alzheimer hastalığına bağlı proteinlerin ekspresyonu]

<https://doi.org/10.1515/tjb-2020-0091>

Received February 22, 2020; accepted July 15, 2020; published online August 28, 2020

Abstract

Objectives: Alzheimer's disease is complex and involves several proteins. Most affected are Tau protein and amyloid precursor protein (APP) which, when cleaved by the enzymes β -secretase (BACE1) and γ -secretase (Nicastrin), yield the amyloid peptide. Although these processes take place mainly in neurons, it is not exclusive of them, as glia cells also contribute to these processes. The objective of

this study was to evaluate the effect of nimesulide, resveratrol and citalopram on C6 (glioma) cells when exposed to Lipopolysaccharide (LPS).

Methods: Expression levels of the proteins APP, BACE1, COX-2, Nicastrin and Tau-p were evaluated by Western-blot and ELISA in C6 cells by effect of LPS, and the drugs citalopram, nimesulide and resveratrol.

Results: It was found that LPS is able to hyperphosphorylate Tau in this cell model and the drugs decrease hyperphosphorylation. We also found that the drugs increase the expression of APP, decrease BACE1 and promote the expression of Nicastrin. COX-2 decreases its expression when nimesulide is used.

Conclusions: Our results suggest that C6 cell line is useful to analyze the effect of pro-inflammatory molecules on tau phosphorylation and APP expression *in vitro*. The beneficial effect on the reduction of tau hyperphosphorylation shown by citalopram, nimesulide and resveratrol should be taken with caution due to the limitations of the present study and further research on these compounds is needed to determine their therapeutic use in neurodegenerative diseases such as Alzheimer's disease.

Amaç: Alzheimer hastalığı karmaşıktır ve birkaç protein içerir. En çok etkilenenler, α -sekretaz (BACE1) ve γ -sekretaz (Nicastrin) enzimleri tarafından parçalandığında amiloid peptidi veren Tau proteini ve amiloid öncü proteinidir (APP). Bu süreçler esas olarak nöronlarda gerçekleşmesine rağmen, glia hücreleri de bu süreçlere katkıda

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bulunduğundan, bunlardan ayrı değildir. Amaç: Bu çalışmanın amacı, LPS'ye maruz kaldığında nimesulid, resveratrol ve sitalopramın C6 (glioma) hücreleri üzerindeki etkisini değerlendirmektir.

Gereç ve Yöntem: APP, BACE1, COX-2, Nicastrin ve Tau-p proteinlerinin ekspresyon seviyeleri, LPS'nin etkisi ile C6 hücrelerinde Western-blot ve ELISA ve sitalopram, nimesulid ve resveratrol ilaçları ile değerlendirildi.

Bulgular: Bu hücre modelinde LPS'nin Tau'yu hiperfosforilat edebildiği ve ilaçların hiperfosforilasyonu azalttığı bulundu. Ayrıca ilaçların APP ekspresyonunu arttırdığını, BACE1'i azalttığını ve Nicastrin ekspresyonunu teşvik ettiğini bulduk. Nimesulid kullanıldığında COX-2 ekspresyonunu azaltır.

Sonuçlar: Sonuçlarımız, pro-enflamatuvar moleküllerin tau fosforilasyonu ve *in vitro* APP ekspresyonu üzerindeki etkisini analiz etmek için C6 hücre hattının yararlı olduğunu göstermektedir. Sitalopram, nimesulid ve resveratrol tarafından gösterilen tau hiperfosforilasyonunun azaltılması üzerindeki yararlı etki, mevcut çalışmanın sınırlamaları nedeniyle dikkatle alınmalı ve bu bileşikler üzerinde, Alzheimer hastalığı gibi nörodejeneratif hastalıklarda terapötik kullanımlarının belirlenmesi için daha fazla araştırmaya ihtiyaç vardır.

Keywords: Alzheimer; citalopram; C6 cells; glia; nimesulide; resveratrol.

Anahtar kelimeler: Alzheimer; nimesulid; resveratrol; sitalopram; C6 hücreleri; glia.

Introduction

Worldwide, approximately 50 million people are affected by dementia, with Alzheimer's disease (AD) being the most common, according to the World Alzheimer's Report 2018 [1]. It is characterized by two typical lesions: the formation of amyloid plaques in the extracellular space and intracellular neurofibrillary tangles. Another key process during the development of AD is neuroinflammation, in which pro-inflammatory molecules such as COX-2, interleukin-1, TNF- α , NF- κ B, among others, have a very relevant effect on the development of the disease and are also associated with malfunctioning of microglia and astrocytes [2]. Together, these elements lead to a slow and progressive deterioration of cognitive functions and memory secondary to neuronal dysfunction [3].

Astrocytes are the most abundant glia cells in the Central Nervous System (CNS) and have multiple functions in organizing and maintaining the structure and function of the brain. Evidence suggests that astrocytes dynamically modulate

information processing, signal transmission, and synaptic plasticity. They also release trophic factors, provide metabolic support and regulate the growth of dendrites and axons [4]. Therefore, the correct functioning of neurons requires the support provided by astrocytes. However, when astrocytes are activated, they become hypertrophic, accumulate β -amyloid in their cytoplasm, occasionally lose their ability to express the EAAT-2 protein, all due to their exposure to amyloid plaques, and finally "neglect" their physiological functions which induces neuronal degeneration, loss of synapses, formation of neurofibrillary tangles and neuritic degeneration [5].

Some authors mention that the presence of astrocytes is essential for the induction of phosphorylation of the Tau protein in the presence of the amyloid peptide β in the primary culture of neurons. This induction is mediated by pro-inflammatory molecules [6]. Hyperphosphorylation of Tau protein is not exclusive to the neuron, although it accumulates in larger amounts in the neuron [7]. In AD the presence of phosphorylated Tau has been described as "spines" within the cytoplasm of astrocytes [8] and occasionally phosphorylated Tau protein is found around the cytoplasmic membrane of the astrocyte.

Although there is no treatment that can prevent or cure the disease at the present time, epidemiological studies have linked patients who are exposed to certain drugs for other reasons not related to the treatment of Alzheimer's to a protective factor for developing the disease, which could potentially be used as an adjuvant in neuroinflammatory diseases, including NSAIDs, polyphenols and antidepressants. However, it is not yet completely understood, whether they can actually provide a therapeutic benefit, as there are not enough studies and several of the existing ones are inconclusive. For that reason, we decided to select one group from each of the above-mentioned groups, which are nimesulide, resveratrol and citalopram, respectively, which are briefly described below along with their potential benefits reported so far in Alzheimer's disease.

Nimesulide is a non-steroidal anti-inflammatory drug (NSAID) that selectively inhibits COX-2, which may be useful in the treatment of the neuroinflammatory process [9]. Some epidemiological studies report possible benefits of regular NSAID administration, for example: delayed onset of AD symptoms in identical twins who have received anti-inflammatory therapy [10] or a lower rate of AD in patients with arthritis [11]. The relative risk of developing AD appears to decrease with increasing duration of NSAID use, being greater than 24 months [12].

Resveratrol is a polyphenolic phytoalexin known for its cardioprotective, antitumor and antioxidant effect and its ability to increase the tolerance of neurons to ischemia [13]. It is also considered a phytoestrogen [14] capable of

activating both estrogen receptors α and β with a similar affinity, but less affinity than estradiol [15]. Some authors suggest that it has therapeutic value in neurodegenerative diseases. It reduces the generation of the β -amyloid peptide and the hyperphosphorylation of the Tau protein, as well as its abnormal aggregation in animal models [16]. One clinical trial found a reduction in MMP9 in cerebrospinal fluid, which induces adaptive immunity and modulates the neuroinflammatory process, suggesting that SIRT1 activators such as resveratrol may have therapeutic utility [17]. It has also been reported that in patients with mild cognitive impairment, they show changes in brain metabolism that they interpret as beneficial when correlated with improved performance in cognitive functions such as attention and working memory, however, in this study the sample of patients was very small [18].

Citalopram is a selective serotonin reuptake inhibitor; studies related to this drug have as their main objective to evaluate its efficacy in reducing agitation in Alzheimer's patients [19]. Although chronic treatment with citalopram has not been reported to have any direct effect on microtubule assembly in the cerebral cortex of rats [20], recent studies have identified that it does have a beneficial effect on tau protein hyperphosphorylation; for example, placing rats in social isolation results in increased expression of tau protein hyperphosphorylation in the rat hippocampus, which can be reversed by citalopram [21].

We utilize rat C6 glioma cells as a model to test the efficacy of the drugs, although it shows certain limitations compared to primary astrocyte culture, it can significantly reproduce the behavior of astrocytes and their ability to respond to pro-inflammatory stimuli such as Lipopolysaccharide (LPS) [22]. For the induction of inflammation in this cellular model, we use LPS. Lipopolysaccharide is a pro-inflammatory agent found in the cell membrane of gram-negative bacteria, it acts through the TLR-4 membrane receptor and can exacerbate the pathology of proteins involved in neurodegenerative processes such as Alzheimer's disease.

The aim of the study is to determine the effect of citalopram, nimesulide and resveratrol on the expression of COX-2, APP, BACE1, nicastrin and hyperphosphorylation of Tau proteins in C6 cells when they are previously stimulated with LPS.

Methodology

C6 cell culture

The rat glioma cell line C6 (ATCC, CCL-107) passage 6, mycoplasma-free, was used as an experimental model as

they are classified as part of the astrocyte lineage, both in neurochemical parameters and in cellular mechanisms. They were maintained in the F-12 (MF-12) medium at 37 °C in a modified atmosphere with 5% CO₂. Cells were seeded in 69.5 cm² wells at a density of 7×10^5 cells and cultured in 3 mL of F-12 medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin for 24 h, and then replaced with MF-12 with 1% penicillin/streptomycin without FBS.

The LPS was used at a concentration of 1 µg/mL without any toxic effects. Therefore, the treatments were divided into the following groups: vehicle, cells are exposed only to DMSO at 0.1%; in which the cells will not be exposed to LPS nor to pharmacological treatment; a group with exposure to 1 µg of LPS per milliliter on average for 6 h and another group for 24 h, without pharmacological treatment; the groups with pharmacological treatment are subdivided into two groups: (a) Without exposure to LPS and (b) with exposure to 1 µg of LPS per milliliter on average for 6 h and they are the following based on the active principle used: Nimesulide to 20 µM, Resveratrol to 25 µM and Citalopram to 40 µM, all for 24 h.

MTT assays and treatments

In order to select the doses used in the study, a bioassay with MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazol bromide, Sigma M2128), was carried out to evaluate the cytotoxicity of the drugs used and to choose the maximum concentration that does not show a statistical difference with respect to the control group with cell arrest and vehicle. 25,000 cells were seeded per well in plates of 96 wells, with MF-12 with SFB for 24 h to allow the adherence of the cells. Subsequently, C6 cells were exposed for another 24 h to four different doses of nimesulide (Sigma, N1016): 5–10–20–40 µM; resveratrol (Sigma, R5010): 12.5–25–50–100 µM; and citalopram hydrobromide (Sigma, C-7861): 10–20–40–80 µM; the control group consisted of cells with MF-12 without SFB and DMSO (vehicle). After 24 h the culture medium was removed and 50 µL of MTT was added in PBS (concentration of 5 mg/mL PBS) for 4 h. Subsequently the PBS was removed with MTT, and 200 µL of DMSO were added, homogenized and the solution was transferred to a reading plate of 96 wells and finally the absorbance was measured at 595 nm.

Western blot

Proteins were evaluated by western blot analysis, using specific antibodies (Santa Cruz Biotechnology, Inc) for

APP and β -amyloid (SC28365), the enzyme COX-2 (SC376861), hyperphosphorylated tau (SC101813), BACE1 (SC33711) and Nicastrin (SC25648), GAPDH (SC25778) was used as a control. For protein extraction, 500 μ L of cold PBS were added to each well from a plate of six wells to wash the cells from the culture medium, 200 μ L of the cell lysis buffer (stock: NP-40 at 1%, Glycerol at 10%, NaCl 137 mM, Tris-HCl 20 mM, pH=8, H₂O, protease inhibitor), was incubated for 10 min at 25 °C with gentle agitation and the monolayer was detached by Scraping. Protein quantification (Bio-Rad Protein Assay Dye Reagent Concentrate #5000006) was done by Bradford's method. Finally, the density of the bands was quantified using the Gel-+Doc+gel documentation system and image Lab software (Biorad).

Enzyme-linked immunosorbent assay (ELISA)

Proteins that are expressed in small amounts, as well as proteins released into the culture medium, were evaluated by an enzyme-linked immunosorbent assay (ELISA). The sandwich ELISA technique was performed, which is 2–5 times more sensitive than conventional antigen-binding ELISA. No commercial kit was used, so the measurements are not given as concentration of the protein, instead absorbance was considered since a chromogenic substrate is used (Phosphatase Substrate 5 mg Tablets S0942-50tab). The results of Figure 1A, B, (Supplementary Material) given in nanometers correspond to the absorbance measured at 415 nm, not to the concentration.

ELISA details: First it was incubated in a 96-well flat-bottomed plate, 100 μ L of buffer (consisting of 4.5 mL of carbonate at 0.2 M and 8 mL of bicarbonate 0.2 M, calibrated to 50 mL with bi-distilled water) together with the primary antibody for 16 h at 4 °C. The excess buffer was removed, and four washes were performed with 200 μ L of TBS-Tween (TBS buffer with 0.5 mL of Tween 20). The blocking of the plate was continued by adding 120 μ L of 1% milk for each well, during 1 h at 37 °C. At the end of the blocking, the milk was removed and again four washes were performed with TBS-Tween.

At the end of the washings, 100 μ L of the previously prepared sample to be analyzed were added (it can be culture medium or cell lysate extract diluted in 1% milk) and incubated for 90 min at 37 °C, then removed and four washings were performed with TBS-Tween. Then, 100 μ L of primary antibody was added per well and incubated at 4 °C for 16 h. At the end of this period, the antibody was removed, and four TBS-Tween washes were performed.

The incubation of the secondary antibody diluted in 1% milk was continued for 1 h at 37 °C. At the end, 100 μ L of fresh phosphatase substrate was added, and the plate was read at 415 nm. The sandwich ELISA technique was used for the following proteins in culture medium: APP (sc28365), phosphorylated tau (sc101813). For total tau (sc32274) the technique will be performed in cell lysate. To choose the appropriate concentration for each antibody, a triplicate curve was made for each one. The concentration of the primary antibody selected was 1/400 for APP and phosphorylated tau (from a 1/200 to 1/12,800 curve). The concentration of the secondary antibody was 1/800 anti-mouse for APP and anti-rabbit for P-tau (from a curve of 1/200 to 1/12,800).

Statistical analysis

All experiments were conducted in triplicate and data are reported as mean $\pm$ SD. To analyze the results, a unidirectional ANOVA was performed with Dunnett's post-hoc test to compare it with the vehicle control group when a statistically significant difference was found. A $p < 0.05$ was considered to show a significant difference between the means.

Results

MTT assay

The MTT bioassay was performed to choose the concentrations of nimesulide, resveratrol and citalopram. The following non-toxic concentrations were used for C6 cells: nimesulide at 20 μ M, resveratrol at 25 μ M and citalopram at 40 μ M (Figure 1).

Effect of nimesulide, resveratrol, citalopram and LPS alone on the expression of COX-2, TAU-P, APP, BACE1 and nicastrin in C6 cell lysate

The effect of the LPS and each drug (nimesulide, resveratrol and citalopram) on the expression of the proteins related to Alzheimer's disease: COX-2, Tau-P, APP, BACE1 and nicastrin, were compared with the vehicle group (0.1% DMSO) in the lysate of C6 cells. We found an increase in the expression of the COX-2 enzyme in the LPS group at 6 h in relation to the vehicle group (DMSO), as well as a slight increase with the drugs resveratrol and citalopram with respect to the vehicle (Figure 2A). The antibody that we used in the experiment detects the phosphorylation of the Tau protein in Ser 262 and a significant increase was observed in the LPS group at 6 h,

Figure 1: MTT bioassay to evaluate cell viability. Comparison of four doses of each drug: N=nimesulide (N01:5 μ M, N02:10 μ M, N03:20 μ M, N04:40 μ M); R=resveratrol (R01:12.5 μ M, R02:25 μ M, R03:50 μ M, R04:100 μ M); C=citalopram (C01:10 μ M, C02:20 μ M, C03:40 μ M, C08:80 μ M); against the control group VH=vehicle (DMSO). The values are expressed as the mean $\pm$ SD. *Concentrations with higher cytotoxicity with respect to the VH group ($p < 0.05$).

which continues to increase at 24 h. There is also a slight significant increase in the presence of the drug citalopram (Figure 2B). Expression of APP protein was increased by the effect of LPS at 24 h as well as with the drugs nimesulide and resveratrol, although an increase at 6 h with LPS was not statistically significant (Figure 2C). In relation to BACE1 protein, we did not find any statistical difference between the different groups compared to the vehicle (Figure 2D). Finally, expression of nicastrin protein was decreased by the effect of all three drugs and the 24-h LPS, relative to the vehicle (Figure 2E).

Effect of nimesulide, resveratrol and citalopram on the expression of COX-2, TAU-P, APP, BACE1 and nicastrin proteins in lysate of C6 cells previously stimulated with LPS

Following independent analysis of the effect of the drugs, we analyzed the effect of the drugs on the expression of the COX-2 protein when the cells were previously stimulated with LPS for 6 h (Figure 3A). Figure 3F shows the WB where the COX-2 protein is identified, it can be seen that in a basal form the C6 cells express relatively low levels of COX-2. Expression levels of the COX-2 enzyme increased after exposure to LPS for 6 h, however its effect decreased after 24 h. When C6 cells were treated with LPS for 6 h and then nimesulide or citalopram was administered, levels decreased significantly from the LPS-treated group for 6 h and normalized to the control group. Resveratrol was the only drug that did not normalize COX-2 expression with respect to the vehicle (Figure 3A). We can observe a relatively weak phosphorylation of the Tau protein in the C6 cells of the vehicle group and its notable increase with exposure to LPS (Figure 3B). Regarding the levels of expression of phosphorylated tau protein, we found that the significant increase after stimulation of C6 cells with LPS for 6 h continues to increase at 24 h with respect to the vehicle group, in contrast to the expression of COX-2

protein which normalizes at 24 h. In addition, it is demonstrated that Tau hyperphosphorylation is significantly reduced by the effect of the three drugs, mainly nimesulide and resveratrol (Figure 3F).

In the WB, where the APP protein was identified in the cell lysate, it was observed that its normal expression was relatively high (Figure 3F). Although the expression levels of APP increased slightly in the LPS group over 6 h compared to the control group, there was no statistically significant difference. A significant increase was only observed when C6 cells were treated with LPS for 24 h and with nimesulide + LPS treatment (Figure 3C). It should be noted that the level of APP in the resveratrol + LPS group was similar to that of the vehicle group, in contrast to the effect of resveratrol when it acted independently on LPS (Figure 2C).

Figure 3F shows the WB where the BACE1 protein was identified. Expression levels of BACE1 showed that there was no change in expression when C6 cells were treated with LPS. On the contrary, its expression decreased significantly when the cells were stimulated with LPS and subsequently nimesulide was added with respect to the vehicle group. No other effect was observed with the other drugs (Figure 3D).

The Nicastrin protein is a component of the protease γ -secretase, which is expressed in C6 cells as seen in the WB in Figure 3F. In our results we found that Nicastrin expression levels are not affected by C6 cell exposure to LPS, similar to BACE1. When the combination of citalopram + LPS was exposed, a slight increase in expression was observed. While nimesulide + LPS slightly decreased the expression of Nicastrin (Figure 3E).

Effect of nimesulide, resveratrol and citalopram on the release of sAPP and TAU-P proteins into the culture medium of C6 cells previously stimulated with LPS

To analyze the soluble proteins APP and TAU-P released in the extracellular space, the culture medium of the C6 cells was analyzed under the different conditions already

Figure 2: Effect of LPS, nimesulide (Nim), resveratrol (Res) and citalopram (Cit), on the expression of COX-2, Tau-P, APP, BACE1 and Nicastrin in C6 cell lysate, $n=3$. (A) Expression of COX-2. (B) Tau-P. (C) APP. (D) BACE1. (E) Nicastrin. The values are expressed as the mean $\pm$ SD. The differences are in relation to the vehicle group: * $p<0.05$, ** $p<0.01$.

mentioned, using the sandwich ELISA technique. Regarding the absorbance levels of sAPP in the culture medium, a statistically significant difference was found between the citalopram group without LPS and the vehicle group. No alteration in sAPP levels was observed after exposure of cells to LPS (Figure 1A, Supplementary Material). An ELISA test was also performed to evaluate the excretion of phosphorylated Tau protein to the culture medium, in which differences were found between the citalopram group without previous stimulus with LPS in relation to the vehicle group, no alteration was observed in relation to the stimulus with LPS (Figure 1B, Supplementary Material). In both ELISA results, citalopram was the only one able to increase the release of sAPP and TAU-P into the extracellular space.

Discussion

The neuroinflammatory process is carried out by astrocytes and microglia. In the case of astrocytes, they are involved in the generation of Tau-P and the release of APP into the extracellular environment during a neuroinflammatory process [23].

An anti-inflammatory treatment could be relevant to prevent neurodegenerative disorders such as AD.

In the present study we analyzed three drugs: nimesulide, resveratrol and citalopram, individually, in a model of C6 cells which were exposed to LPS. LPS is a potent pro-inflammatory agent that does not affect cell viability [24]. LPS acts increasing reactive oxygen species as well as direct activation of astrocytes and microglia [25]. LPS acts in a similar way to the beta-amyloid peptide, because it activates NF- κ B in the dose-response relationship in astrocytes [26].

The results obtained confirm that LPS significantly increases the levels of hyperphosphorylated Tau (Ser 262) in C6 cells with gradual increase after 24 h. Similar results have been demonstrated in rTg4510 transgenic mice [27]. LPS in C6 cells has been shown to increase levels of TNF- α [28], caspase 3 [29], GFAP [29], nitric oxide, prostaglandin E2 [28], IL-1 β [30], NF- κ B [31] and COX-2 [28]. This makes it a good model to study Tau and neuroinflammatory processes.

It was found that both citalopram and nimesulide are able to decrease the levels of hyperphosphorylated tau to normal values, an effect that is not achieved with resveratrol. Resveratrol was also shown to reduce tau hyperphosphorylation in serine 396 [32] in the hippocampus

Figure 3: Effect of LPS and drugs + LPS on the expression of COX-2, Tau-P, APP, BACE1 and Nicastrin in C6 cell lysate, $n=3$. (A) Expression of COX-2. (B) Tau-P. (C) APP. (D) BACE1. (E) Nicastrin. (F) WB image of proteins COX-2, Tau-P, APP, BACE1 and Nicastrin. The values are expressed as the mean $\pm$ SD. The differences are in relation to the vehicle group: * $p<0.05$, ** $p<0.01$.

cultures, as in this study we demonstrated a decrease in tau hyperphosphorylation in (Ser 262). Studies have also shown that nimesulide by inhibiting COX-2 could indirectly decrease tau phosphorylation levels [33].

When cells were exposed to nimesulide, an increased expression of APP is shown in the C6 cell lysate, this result could be due to a decrease in APP processing due to inhibition of BACE1 and reduced gamma-secretase function. However, we observed a slight increase in Nicastrin expression when treating cells with citalopram. This result may be consistent with Cirrito et al. [34], where they show that serotonin reuptake inhibitors decrease β -amyloid levels in the plaques of transgenic mice and in humans.

When we exposed the cells to resveratrol, we found no significant effect on the secretases so we propose that the effect of resveratrol is through the activation of proteosomes, which metabolize to APP through an alternate pathway to amyloidegenic and that a decrease in proteosome activity occurs in brains with Alzheimer's disease [35]. As for the effect of non-steroidal anti-inflammatory drugs on APP processing, ibuprofen has been reported to inhibit gamma-secretase, thereby reducing β -amyloid production by an independent mechanism of COX-2 [36] in our study we found that nimesulide is capable of

decreasing BACE1 expression, but not citalopram and resveratrol.

Astrocytes, the most abundant type of glia in the brain, have the primary function of providing metabolic and trophic support to neurons and modulate synaptic activity. The interaction of neurons with astrocytes is necessary for the release of trophic and survival factors of neurons. Studies have shown that apoptosis in astrocytes can contribute to the pathogenesis of Alzheimer's disease due to a reactive astrocytosis where a large amount of protein β -amyloid is released to the extracellular space [37]. It is important to avoid or at least control astrocytosis so that apoptosis is not generated by astrocytes that could be the cause of Alzheimer's disease.

We here demonstrate that the drugs decrease Tau-P and that it could be released into the space between neurons and glia. Tau-P could probably be added by being part of amyloid plaques, as demonstrated in the endotoxemia study where β -amyloid and Tau-P formation is induced, and that is could be distributed throughout the brain over time [38]. Treatment with citalopram induced a greater release of phosphorylated tau protein and sAPP into the culture medium detected by ELISA, other studies have reported that citalopram increases sAPP secretion in primary

Table 1: Effect of drugs on protein expression in C6 cells treated with LPS.

Protein expression	Drugs + LPS		
	Nimesulide	Resveratrol	Citalopram
COX-2	Normal	Up	Normal
Tau-P	Normal	Up	Normal
APP	Up	Normal	Normal
BACE1	Down	Normal	Normal
Nicestrin	Down	Normal	Up

culture of rat neurons by 3.4 times, PKC levels are also increased, not being a future substrate for β -secretase consequently there is a decrease in the production of β -amyloid, so it is considered a non-amyloidogenic pathway of the APP metabolism [39], however there are no reports on the release of phosphorylated Tau, it is likely to share a common mechanism by which the exocytosis of multiple proteins is increased. The greatest release of vesicles into extracellular space is probably through the activation of the synaptophysin protein [40]. Therefore, citalopram probably does not have a direct effect on the hyperphosphorylation of the Tau protein, since the decrease of this protein in the cell lysate is due to an increase in its release into the culture medium. It could also avoid a neuroinflammatory process due to the use of nimesulide that could eventually lead to the generation of amyloid plaques, typical of Alzheimer's disease (Figure 2, Supplementary Material).

Conclusion

Our results suggest (Table 1) that the C6 cell line is useful to analyze the effect of pro-inflammatory molecules on tau phosphorylation and APP expression *in vitro*, as well as to study the molecular mechanisms that act in this process. As an economic model and easy standardization in comparison with animal models and clinical trials. The most important limitation of this study is that the results cannot be extrapolated to humans due to the complexity of an organism. Therefore, although the action of the drugs citalopram, nimesulide and resveratrol have a positive effect on this model, more studies are needed to demonstrate their real benefit for the treatment of neurodegenerative diseases associated with a neuroinflammatory process such as Alzheimer's disease.

Research funding: We thank CONACYT, for the support given to JAMD, for the scholarship for doctoral studies.

Author contribution: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Conflict of interest: Authors have no conflict of interest.

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Supplementary Material: The online version of this article offers supplementary material (<https://doi.org/10.1515/tjb-2020-0091>).