

Palmitoylethanolamide blunts A β ₄₂-induced astrocyte activation and improves neuronal survival in primary mouse cortical astrocyte-neuron co-cultures.

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Running title: PEA and cortical astrocyte-neuron co-cultures

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Abstract

Background

Based on the pivotal role of astrocytes in brain homeostasis and the strong metabolic cooperation existing between neurons and astrocytes, it has been suggested that astrocytic dysfunctions might cause and/or contribute to neuroinflammation and neurodegenerative processes. Therapeutic approaches aimed both to neuroprotection and to neuroinflammation reduction may prove particularly effective in slowing the progression of these diseases. The endogenous lipid mediator palmitoylethanolamide (PEA) displayed neuroprotective and anti(neuro)inflammatory properties, and demonstrated interesting potential as a novel treatment for Alzheimer's disease.

Objective and Methods

We firstly evaluated whether astrocytes could participate in regulating the A β ₄₂-induced neuronal damage, by using primary mouse astrocytes cell cultures and mixed astrocytes-neurons cultures. Furthermore, the possible protective effects of PEA against A β ₄₂-induced neuronal toxicity have also been investigated by evaluating neuronal viability, apoptosis and morphometric parameters.

Results

The presence of astrocytes pre-exposed to A β ₄₂ (0.5 μ M; 24 hours) induced a reduction of neuronal viability in primary mouse astrocytes-neurons co-cultures. Furthermore, under these experimental conditions, an increase in the number of neuronal apoptotic nuclei and a decrease in the number of MAP-2 positive neurons were observed. Finally, astrocytic A β ₄₂ pre-exposure induced an increase in the number of neurite aggregations/100 μ m as compared to control (*i.e.* untreated) astrocytes-neurons co-cultures. These effects were not observed in neurons cultured in the presence of astrocytes pre-exposed to PEA (0.1 μ M), applied 1 hour before and maintained during A β ₄₂ treatment.

Conclusion

Astrocytes contribute to A β ₄₂-induced neurotoxicity and PEA, by blunting A β ₄₂-induced astrocyte activation, improved neuronal survival in mouse astrocyte-neuron co-cultures.

Key words: Alzheimer's disease; Cell viability; MAP-2 immunoreactivity; Hoechst 33258.

Introduction

Alzheimer's disease (AD) is a devastating, multifactorial pathology causing a progressive and irreversible deterioration of cognitive performance. The neuropathological hallmarks of AD comprise "positive" lesions like amyloid plaques and cerebral amyloid angiopathy, neurofibrillary tangles, and glial responses, along with "negative" lesions such as neuronal and synaptic loss [1]. In the last decades, the neuron-centered view of AD neuropathology has changed, and neuroglia is now considered to be involved in the onset and progression of AD [2-5]. Indeed, the presence of activated microglia and reactive astrocytes, sustaining ongoing neuroinflammatory processes during AD progression, are now well-documented both *in vivo* and *in vitro* [3, 6-7]. Astrocytes outnumber microglia in the brain and their activation seems to last longer than in other glial cells. This supports the view that these cells may have a more important and sustained role over microglia and represent significant sources of damage during neuroinflammation in AD [8-10]. In this context, it has been reported that impairments in astrocytic functions are deeply involved in neuronal dysfunction, and could contribute to neurotoxicity observed in the neurodegenerative process of AD [11-12]. Excessive and prolonged astrocyte activation contributing to a sustained neuroinflammatory process might, therefore, alters the neuron/astrocyte cooperation, leading to deleterious effects on neurons and contributing to the expression of the hallmark lesions of AD. Thus, a therapeutic approach aimed at reducing both neuroinflammation and neurodegeneration may prove effective in slowing the progression of the disease.

The endogenous lipid mediator palmitoylethanolamide (PEA) is currently used in the clinic for its anti-neuroinflammatory and analgesic properties [13]. In the central nervous system (CNS), PEA is produced by neurons, microglia and astrocytes, and exerts a local anti-injury function through a down-modulation of mast cells and by protecting neurons from excitotoxicity [14]. The involvement of astrocytes, microglia and keratinocytes in PEA anti-inflammatory actions has also been proposed [13, 15-16]. The pharmacology of PEA is quite complex: although PPAR- α receptors are the molecular targets directly mediating some of the neuroprotective, anti-neuroinflammatory and analgesic effects of PEA [13-14, indirect mechanisms of action for this compound have also been demonstrated. For instance, indirect endocannabinoid mechanisms have been implicated in some PEA-induced effects, leading to the theory of the so-called 'entourage' effect [13]. PEA displayed neuroprotective properties in several *in vitro* and *in vivo* animal models of AD [17-22]. Furthermore, we recently demonstrated that PEA exerts differential protective effects against β -amyloid (A β ₄₂)-

induced toxicity in primary cultures of cortical neurons or astrocytes from wild-type (non-Tg) and the triple-transgenic murine model of AD (3xTg-AD) mice [23]. Finally, it has been lately reported that PEALut, containing PEA and the antioxidant flavonoid luteolin (Lut; 10:1 by mass), induced an improvement of cognitive performances in a patient affected by mild cognitive impairment [24], thus suggesting that the compound might exert some beneficial effects on cognition also in humans.

In the present study, we firstly evaluated whether astrocytes could participate in regulating the A β ₄₂-induced neuronal damage, by using primary mouse astrocytes cell cultures and astrocytes-neurons co-cultures. Secondly, the possible protective effects of PEA against A β ₄₂-induced neuronal toxicity have also been investigated by evaluating neuronal viability, apoptosis and morphology.

Materials and Methods

Animals

C57Bl/6 mice, 25-30 g (Charles-River, Milan, Italy), were group-housed (8-10 mice per cage; floor area per animal was 80 cm²; minimum enclosure height was 12 cm) on a 12:12-hour light-dark cycle (light period from 7:00 a.m. to 7:00 p.m.), temperature of 20-22°C, humidity of 45-55% and were provided with ad libitum access to food (Diet 4RF25 GLP; Mucedola, Settimo Milanese, Milan, Italy) and water. The experimental protocols performed in the present study were in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines and the new European Communities Council Directive of September 2010 (2010/63/EU) a revision of the Directive 86/609/EEC. Moreover experimental protocols were approved by the Italian Ministry of Health (authorization number: 987/2016-PR). Efforts were made to minimize the number of animals used to reduce their discomfort.

Primary cultures of cerebral cortical neurons

Primary cultures of mouse cortical neurons were prepared from embryonic day 18 (ED18) on the basis of the experimental procedure previously described for rat cortical neurons [25]. Briefly, cortices free of meninges were dissociated in 0.025% (w/v) trypsin at 37°C. The tissue fragments were dissociated mechanically by repeated gentle pipetting through wide- and narrow-bore fire-polished Pasteur pipettes in culture medium [Neurobasal medium

(Gibco, Grand Island, NY, USA) supplemented with 0.1 mM glutamine (Sigma Chemical Co., St. Louis, MO, USA), 10 µg/ml gentamicin (Sigma Chemical Co.) and 2% B27® Supplement (50X), serum free (Gibco®)]. Cells were counted and then plated on glass coverslips in a poly-L-lysine (5µg/ml)-coated multiwells (24 wells; Nunc A/S, Roskilde, Denmark) at a density of 200,000 cells per well. Cultures were grown at 37°C in humidified atmosphere, 5% CO₂/95% air. The cultures were used in experiments after 8 days of *in vitro* incubation (days *in vitro*: DIV).

Primary cultures of cerebral cortical astrocytes

Primary cultures of cerebral cortical astrocytes were obtained from newborn mice (1-2 days old) and cultured as previously described [18, 23]. Briefly, cerebral cortices were removed and dissociated by mild trypsinization at 37°C, followed by mechanical trituration to obtain single cells. Cells were suspended in culture medium [DMEM, 5% inactivated fetal bovine serum (Gibco), 100 IU/ml penicillin and 100 µg/ml streptomycin (all from Sigma-Aldrich, Milan, Italy)] and then seeded in 75-cm² flasks at a density of 3×10⁶ cells/flask. The cells were incubated at 37°C in humidified atmosphere, 5% CO₂/95% air. Culture medium was replaced after 24 hours and again twice weekly until astrocytes were grown to form a monolayer firmly attached to the bottom of the flask (12 or 14 days after dissection). At cell confluence, flasks were vigorously shaken to separate astrocytes (which remained adherent in the bottom of the flasks) from microglia and oligodendrocytes (which floated on the supernatant). Collected astrocytes were counted and then plated on poly-L-lysine (5 µg/ml)-coated Petri dish (35 mm diameter) at a density of 1,500,000 cells per dish (Nunc A/S, Roskilde, Denmark). The purity of the cells in culture was tested with monoclonal anti-glial fibrillary acidic protein (GFAP), and only cultures with more than 95% GFAP-positive cells were employed for the experiments.

Astrocyte-neuron co-cultures and treatment protocol

Astrocyte-neuron co-cultures were obtained on the basis of the experimental procedure described by Allaman et al. [11].

Astrocytes cultured in dish containing DMEM were treated with A β ₄₂ (0.5 μ M) (Tocris Bioscience, Bristol, UK) for 24 hours. When required, 0.1 μ M PEA was added 1 hour before A β ₄₂ and maintained in contact with the cells throughout the whole A β ₄₂ exposure (24 hours). After this period, A β ₄₂ and PEA were removed by medium aspiration and the astrocytes were rinsed with fresh DMEM and then placed in supplemented Neurobasal Medium for 4 hours. At the end of this washing period, astrocyte-neuron co-cultures were initiated by the gentle transfer of primary cortical neurons grown on coverslips on the top of the astrocyte layer cultured in 35 mm dish (neurons facing up). 24 hours after co-cultures initiation, neuronal viability, MAP-2 immunoreactivity, nuclear staining and the morphometric parameter were evaluated. It is worth noting that, under the present experimental conditions, neurons were never directly in contact with A β ₄₂ and/or PEA. The concentrations of A β ₄₂ and PEA were chosen on the basis of previous results [18, 23, 26].

Neutral red uptake assay

The neutral red uptake assay was used to assess neuronal viability as previously described [19, 27] with slight modifications. Briefly, 24 hours after astrocyte-neuron co-culture initiation, the dishes were incubated for 2 hours at 37°C with a neutral red working solution (50 μ g/ml in 1X PBS not containing calcium and magnesium, Sigma-Aldrich, MO, USA). The cells were then washed with phosphate-buffered saline (PBS; 0.1 M) in order to remove the staining solution. Then, the glass coverslips with cortical neurons were removed from the 35 mm dishes containing the astrocytes and positioned in 24-multiwell plates with neurons facing up. The dye was removed from each well through a destaining solution (ethanol:deionized water:glacial acetic acid, 50:49:1 v/v/v). The absorbance was read at 540 nm, using a Tecan's Sunrise absorbance microplate reader. The values of treated cells were referred to control non-exposed cultures, and expressed as percentage variation.

Nuclear staining with Hoechst 33258

24 hours after the preparation of astrocyte-neuron co-cultures, the coverslips with cortical neurons were removed and rinsed twice with PBS (0.1 M), and then cells were fixed in 4% paraformaldehyde, and incubated for 20 min at room temperature with Hoechst 33258 (1 g/ml in 0.1 M PBS). After rinsing with PBS, coverslips were mounted on slides with a

solution containing 50% glycerol in 0.044 M citrate, 0.111 M phosphate buffer, pH 5.5, and visualized under a fluorescence microscope (Nikon Microphot FXA). The percentage of cells showing chromatin condensation (fragmented nuclei) was quantified by counting >3000 cells under each experimental condition (5 randomly selected fields per coverslip, 9-18 wells per experimental group, 5 independent experiments).

MAP-2 immunoreactivity

The MAP-2 immunocytochemistry was assessed to analyze neuronal morphology. Briefly, 24 hours after astrocyte-neuron co-culture preparation, glass coverslip with cortical neurons facing up, were removed from the dishes with cortical astrocytes and positioned in 24-multiwell plates. Cells were rinsed in 0.1 M PBS and then fixed with 4% paraformaldehyde in Sorensen's buffer 0.1 M, pH 7.4 for 20 min. After rinsing in PBS (0.1 M; 3 times for 5 min each), the cells were incubated overnight at 4°C in 0.3% Triton X-100/PBS solution containing the primary antibody-microtubule-associated protein 2 (MAP-2) (1:1000 dilution Chemicon, Temecula, CA). Neurons were then washed 3 times with PBS (0.1 M) and incubated for 60 min at room temperature with the secondary antibody, rhodamine-conjugated anti-rabbit antibody (1:100 dilution Chemicon, Temecula, CA). Then the cells were washed in PBS and mounted in glycerol and PBS (3:1 v/v) containing 0.1% 1,4-phenylenediamine and examined using a Nikon Microphot FXA microscope. For the cell counts, 5 separate non-overlapping fields were randomly chosen in each coverslip and the images were taken using the 20x objective.

Morphometric neuronal analysis

The investigation of morphological parameters (cell body area, length of the longest dendrite, number of dendritic branches per neuron, number of neurites per neuron and MAP-2 dendrite aggregations) was performed with Nikon Microphot FXA microscope, using 40x magnification objective on 5 randomly chosen microscopic fields in each coverslip. Then the number of MAP-2 aggregations was counted and referred to 100 µm of dendritic length. Morphometric neuronal analysis was carried using Image-Pro Plus 4.1 software [25].

Statistical Analysis

Results are expressed as means \pm standard error of mean. The statistical analysis was carried out by analysis of variance (ANOVA) followed by the Newman-Keuls test for multiple comparisons. $p < 0.05$ was the accepted level of significance.

Results

Impact of astrocytic $A\beta_{42}$ pre-exposure, in the presence or in the absence of PEA, on neuronal viability as evaluated in cortical astrocyte-neuron co-cultures

We firstly investigated the impact of astrocytic $A\beta_{42}$ pre-exposure on neuronal viability. To this end, cortical astrocyte cultures were pretreated with $A\beta_{42}$ (0.5 μM ; 24 hours) and, after changing the culture medium (to remove $A\beta_{42}$), neurons were co-cultured for another 24 hours period, after which cell viability was assessed.

In the presence of astrocytes pre-exposed to $A\beta_{42}$, neuronal viability was significantly decreased (Fig. 1) when compared to that observed in control (*i.e.* untreated) astrocyte-neuron co-cultures. This effect was not observed in neurons cultured in the presence of astrocytes pre-exposed to PEA (0.1 μM), applied 1 hour before, and maintained during, $A\beta_{42}$ treatment (Fig. 1).

Impact of astrocytic $A\beta_{42}$ pre-exposure, in the presence or in the absence of PEA, on neuronal MAP-2 immunoreactivity as evaluated in cortical astrocyte-neuron co-cultures

As shown in Fig. 2, cultured cortical neurons from control astrocyte-neuron co-cultures presented a homogeneous MAP-2 immunoreactivity in cell bodies and dendrites, demonstrating a healthy appearance and their ability to develop a complex neuronal network, mainly characterized by highly arborized dendritic trees. In the presence of astrocytes pre-exposed to $A\beta_{42}$ (0.5 μM ; 24 hours) a dishomogeneous distribution of MAP-2 immunoreactivity along the neurites, suggesting neurite fragmentation, was observed (Fig. 2). To quantify the effect of astrocytic $A\beta_{42}$ pre-exposure on neuronal MAP2 immunoreactivity, the number of vital MAP-2 immunostained cultured cortical neurons obtained from astrocyte-neuron co-cultures was determined. As showed in Fig. 3, the number of MAP-2 positive neurons was significantly reduced in the co-cultures where the astrocytes were pre-exposed to $A\beta_{42}$, as compared to control co-cultures. The decrease of vital MAP-2 immunostained

cultured cortical neurons induced by pre-exposure of astrocytes to A β ₄₂ was abolished by astrocytic pre-treatment with PEA (0.1 μ M; 1 hour before A β ₄₂), by itself ineffective on neuronal MAP2 immunoreactivity (Fig. 3).

Impact of astrocytic A β ₄₂ pre-exposure, in the presence and in the absence of PEA, on neuronal morphometric parameters as evaluated in cortical astrocyte-neuron co-cultures

By using MAP-2 immunofluorescence, a morphometric analysis was carried out to quantitatively characterize the neurite maturation in cultured cortical neurons from control and astrocyte A β ₄₂ pre-exposed co-cultures. No significant differences in cell body area, length of the longest dendrite, number of dendritic branches per neuron and number of neurites per neuron was observed following astrocytic A β ₄₂ pre-exposure (*data not shown*). On the contrary, astrocytic A β ₄₂ pre-exposure induced an increase in the number of neurite aggregations/100 μ m as compared to control co-cultures (Fig. 4). The astrocytic pre-treatment with PEA (0.1 μ M; 1 hour before A β ₄₂), by itself ineffective, counteracted the astrocytic A β ₄₂ pre-exposure induced increase of aggregation/100 μ m number (Fig. 4).

Impact of astrocytic A β ₄₂ pre-exposure, in the presence and in the absence of PEA, on apoptotic neuronal death as evaluated in cortical astrocyte-neuron co-cultures

The specific DNA stain, Hoechst 33258, was used to assess chromatin structure alteration, considered as an index of apoptotic neuronal death, in cultured cortical neurons obtained from astrocyte-neuron co-cultures. Representative fluorescence photomicrographs of cultured cortical neurons from control astrocyte-neuron co-cultures were reported in Fig. 5. These neurons exhibited nuclei with a diffuse and homogeneous chromatin staining. In contrast, following astrocytic A β ₄₂ (0.5 μ M; 24 hours) pre-exposure, nuclei of cultured cortical neurons from astrocyte-neuron co-cultures showed a variety of abnormal morphologies, caused by highly condensed and fragmented chromatin (Fig. 5).

To quantify the effect of astrocytic A β ₄₂ pre-exposure, the number of apoptotic nuclei in cultured cortical neurons obtained from astrocyte-neuron co-cultures was determined. As shown in Fig. 6, the number of apoptotic nuclei was significantly higher in neurons from co-cultures where the cerebral cortical astrocytes were pre-exposed to A β ₄₂ than in control co-cultures. The astrocytic pre-treatment with PEA (0.1 μ M), by itself ineffective, counteracted the A β ₄₂ pre-exposure induced increase of apoptotic nucleus number (Fig. 6).

Discussion

Astrocytes and neurons constitute a highly specialized functional unit, and currently it is becoming clear that astrocyte function goes further than the well-known neurotrophic support. It is now generally accepted that the interactions between astrocytes and neurons are not only crucial for the physiological brain function, but are also involved in the development/progression of several neurological disorders, including AD, through pathways that include inflammation, oxidative stress, cell signaling, necrosis, and apoptosis [3, 28]. The onset of astrocyte dysfunction could be a crucial factor in the development of neurodegeneration, being astroglia strategically important to preserve a correct and functional connection between the components of the neuronal network [5, 29]. A better understanding of these interactions could be beneficial also in view of the potential development of new therapeutic approaches. In this context, astrocyte-neuron co-cultures represent a valuable *in vitro* experimental model to study the communication between astrocytes and neurons, also under experimental conditions that simulate events occurring in a pathological context, such as AD. Thus, in the present study, this model has been used to evaluate whether astrocytes could participate in A β ₄₂-induced neuronal damage.

We previously demonstrated that A β ₄₂ (0.5 μ M; 24 hours) exposure causes reactive astrogliosis and an increase in glutamate levels in mouse cultured cortical astrocytes (Tomasini et al., 2015). Furthermore, reactive astroglia express and release pro-inflammatory mediators interleukin (IL)-1 β , IL-6, tumor necrosis factor- α , interferon- γ , inducible NO synthase (iNOS) and reactive oxygen species (ROS) [5, 9, 30] which induce a long term and self-perpetuating deleterious response [31]. This suggests that reactive astrocytes play a primary role in the pathogenesis of AD. In the present study, we provide evidence that astrocyte modifications have functional consequences for neighbouring neurons, since A β ₄₂-treated astrocytes impaired neuronal viability in a co-culture model. In particular, we demonstrate that astrocytic A β ₄₂ pre-exposure has a negative impact on neuronal viability and morphometric parameters. It is worth noting that in the astrocyte-neuron co-culture model we used, the two different cell populations were not in direct contact with each other. In fact, neurons grown on glass coverslips were transferred facing up to a monolayer of astrocytes previously exposed to A β ₄₂. In this way, neurons have never been directly exposed to A β ₄₂ and were only in contact with the culture medium containing mediators which would potentiate neuronal demise. These results are in line with previous data demonstrating that aggregated forms of A β alter glucose metabolism and oxidative stress status in astrocytes

thus impairing neuronal viability in a similar co-culture preparation, as used in the present study [11].

In the presence of astrocytes pre-exposed to A β ₄₂, a significant decrease in cell viability of cultured neurons was observed, suggesting that astrocytes are able to perpetuate the neurotoxic effects of the peptide. This effect on neuronal survival can be correlated to the reduction in the number of cultured neurons, as showed by MAP-2 immunofluorescence experiments, where the number of positive cells after astrocytic A β ₄₂ pre-exposure was significantly lower than that measured in control astrocyte-neuron co-cultures. Interestingly, these results reproduce the pattern observed in our previous study, where direct neuronal exposure to A β ₄₂ (0.5 μ M; 24 hours) reduced cell viability and the number of MAP-2 immunostained cultured neurons [23]. MAP-2 cytoskeletal protein can influence the development of neurites and their function at synaptic level and consequently modifying synaptic plasticity. The astrocytic A β ₄₂ pre-exposure induced a dishomogeneous distribution of MAP-2 immunoreactivity along the neurites, thus revealing impairments in the neuronal outgrowth. Indeed, the evaluation of morphometric parameters showed an increase in the number of aggregation/100 μ m, suggesting a fragmentation of neuronal network also associated with neurite breakage. On the contrary, the presence of astrocytes pre-exposed to A β ₄₂ did not significantly modify other morphometric parameters like cell body area, length of dendrites and neuritic arborisation, thus suggesting a different vulnerability of these parameters to A β ₄₂ neurotoxic effects.

The biochemical determination of cell viability and the MAP-2 morphological analysis did not allow establishing whether astrocytic A β ₄₂ pre-exposure directly or indirectly induced apoptotic neuronal death. Therefore, the specific DNA stain Hoechst 33258 was used to assess chromatin condensation (*i.e.* an index of apoptosis). An increase of apoptotic nuclei was observed in cultured neurons placed in contact with astrocytes pre-exposed to A β ₄₂. Interestingly, apoptosis was not observed when neuronal mono-cultures were directly exposed to A β ₄₂ (0.5 μ M; 24 hours; Tomasini et al., *unpublished data*). This finding suggests that astrocytes could amplify A β ₄₂-induced neurotoxicity, thus possibly playing a relevant role in AD pathology. In line with the present data, A β ₄₂ exposure induced apoptotic neuronal cell death in mixed neuroglia co-cultures [18]. Interestingly, it has been shown that a localized apoptotic mechanism, defined “synaptic apoptosis” could contribute to a decrease in neuritic development, and this early neurite damage has been associated to the initial cognitive decline in AD [32, 33]. Therefore, it seems likely that apoptotic mechanisms could contribute to the observed reduction of neuronal survival and to the neurite fragmentation

induced by astrocytes pre-exposed to A β ₄₂ and that A β ₄₂-induced glial activation may be responsible for neurotoxicity.

As already reported, neuroinflammation is present during neurodegenerative diseases and the release of pro-inflammatory mediators makes neurons more vulnerable to cytotoxic events, leading to their death. Therefore it is evident that a therapeutic approach aimed to both neuroprotection and the reduction of neuroinflammatory processes could be effective to slow the course of neurodegenerative events. Preclinical and human studies indicate that the endogenous lipid mediator PEA is a therapeutic tool with relevant potential for the treatment of different pathologies characterized by neurodegeneration, neuroinflammation and pain [13]. Furthermore, important variations in endogenous PEA levels were observed in neurological disorders such as multiple sclerosis, neuropathic pain and Huntington's chorea [13, 34]. *In vivo* rodent studies also indicate that PEA displays interesting properties as a novel treatment for AD. For instance, it has been previously demonstrated that PEA, possibly through peroxisome proliferator-activated receptor- α (PPAR- α) activation, reduced the behavioural impairments, lipid peroxidation, iNOS induction and caspase-3 activation in an animal model of AD [17]. Moreover, PEA antagonized the increased transcription and expression of GFAP and S100 β , BACE1 and APP, and phosphorylated τ proteins, in a different AD animal model [20]. Protective effects of PEA against *in vitro* A β ₄₂-induced neurotoxicity have also been demonstrated [18].

In order to further investigate on the putative role of PEA as therapeutic agent in AD, in the present study the effects of the compound against astrocytic A β ₄₂ pre-exposure induced neuronal damages have been investigated in astrocytes-neuronal co-cultures. The results indicate that the pre-treatment of astrocytes with PEA fully counteracted the neuronal cell death, the reduced number of MAP-2 positive cells, the neurite fragmentation and the increase of apoptotic nuclei induced by astrocytic A β ₄₂ pre-exposure. These data are in agreement with our previous study, where PEA displayed protective properties in mouse neuron and astrocyte monocultures exposed, under the same experimental conditions as used in the present study, to the peptide [23]. It seems likely that the neuroprotective effects of PEA are related to its ability to counteract the production and release of pro-inflammatory mediators by astrocytes. In fact, PEA-induced reduction of astrocytic production of pro-inflammatory molecules and cytokine release was shown in an *in vitro* model of A β ₄₂-induced neurotoxicity [26].

Besides A β aggregation, oxidative stress is another hallmark of AD that has been linked to A β toxicity [35, 36], and astrocytes have been proposed to take part in this process, since A β

stimulates ROS production and decreases glutathione levels in these cells [37, 38]. In line with these observations, it has been reported that A β strongly modifies glucose metabolism and oxidative stress status in astrocytes and that these effects are mediated through their binding to scavenger receptors of the class A family and the PI-3 kinase pathway [11]. These modifications induced functional detrimental consequences for neighbouring neurons, since A β -treated astrocytes impaired neuronal viability in a co-culture model. Thus, it cannot be ruled out that PEA pre-treatment also counteracts the A β -induced alterations in astrocyte glucose metabolism, an effect that may contribute to the neuroprotective action displayed by the compound in the present study.

In conclusion, the results obtained in the present study indicate that astrocytes contribute to A β_{42} induced neurotoxicity and that PEA, by blunting A β_{42} -induced astrocyte activation improved neuronal survival in mouse astrocyte-neuron co-cultures. Based on these findings, it can be assessed that PEA exerts neuroprotective effects not only by a direct action at the neuronal level, but also by reducing the deleterious consequences of A β -induced astrocyte dysfunctions. These findings suggest that PEA may be effective in the early AD or when β -amyloid is accumulating and initiating damage in the CNS. Thus, it will be relevant to evaluate the effects of PEA on astrocyte-neuron co-cultures from an animal model of AD. From a translational point of view, it is noteworthy that PEA is already licenced for use in humans and displays a high tolerability and safety profile [39].

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Conflict of Interest

The authors have no conflict of interest to report.

References

- [1] Serrano-Pozo A, Frosch MP, Masliah E, Hyman BT (2011) Neuropathological alterations in Alzheimer disease. *Cold Spring Harb Perspect Med* **1**,:a006189.
- [2] Lyman M, Lloyd DG, Ji X, Vizcaychipi MP, Ma D (2014) Neuroinflammation: the role and consequences. *Neurosci Res* **79**, 1-12.
- [3] Steardo L Jr, Bronzuoli MR, Iacomino A, Esposito G, Steardo L, Scuderi C (2015) Does neuroinflammation turn on the flame in Alzheimer's disease? Focus on astrocytes. *Front Neurosci* **9**, 259.
- [4] Hong S, Beja-Glasser VF, Nfonoyim BM, Frouin A, Li S, Ramakrishnan S, Merry KM, Shi Q, Rosenthal A, Barres BA, Lemere CA, Selkoe DJ, Stevens B (2016) Complement and microglia mediate early synapse loss in Alzheimer mouse models. *Science* **352**, 712-716.
- [5] Pekny M, Pekna M, Messing A, Steinhäuser C, Lee JM, Parpura V, Hol EM, Sofroniew MV, Verkhratsky A (2016) Astrocytes: a central element in neurological diseases. *Acta Neuropathol* **131**, 323-45.
- [6] Bouvier DS, Murai KK (2015) Synergistic actions of microglia and astrocytes in the progression of Alzheimer's disease. *J Alzheimer's Dis* **45**, 1001-14.
- [7] Acosta C, Anderson HD, Anderson CM (2017) Astrocyte dysfunction in Alzheimer disease. *J Neurosci Res* doi: 10.1002/jnr.24075 (*in press*).
- [8] Li C1, Zhao R, Gao K, Wei Z, Yin MY, Lau LT, Chui D, Yu AC (2011) Astrocytes: implications for neuroinflammatory pathogenesis of Alzheimer's disease. *Curr Alzheimer Res* **8**, 67-80.
- [9] Zhao J, O'Connor T, Vassar R (2011) The contribution of activated astrocytes to A β production: Implications for Alzheimer's disease pathogenesis. *J. Neuroinflammation* **8**, 150.
- [10] Avila-Muñoz E, Arias C (2014) When astrocytes become harmful: functional and inflammatory responses that contribute to Alzheimer's disease. *Ageing Res Rev* **18**, 29-40.
- [11] Allaman I, Gavillet M, Bélanger M, Laroche T, Viertl D, Lashuel HA, Magistretti PJ (2010). Amyloid-beta aggregates cause alterations of astrocytic metabolic phenotype: impact on neuronal viability. *J Neurosci* **30**, 3326-38.
- [12] Allaman I, Bélanger M, Magistretti PJ (2011) Astrocyte-neuron metabolic relationships: for better and for worse. *Trends Neurosci* **34**, 76-87.
- [13] Petrosino S, Di Marzo V (2017) The pharmacology of palmitoylethanolamide and first data on the therapeutic efficacy of some of its new formulations. *Br J Pharmacol* **174**, 1349-1365.

- [14] Mattace Raso G, Russo R, Calignano A, Meli R (2014) Palmitoylethanolamide in CNS health and disease. *Pharmacol Res* **86**, 32-41.
- [15] Luongo L, Guida F, Boccella S, Bellini G, Gatta L, Rossi F, de Novellis V, Maione S (2013) Palmitoylethanolamide reduces formalin-induced neuropathic-like behaviour through spinal glial/microglial phenotypical changes in mice. *CNS Neurol Disord Drug Targets* **12**, 45-54.
- [16] Guida F, Luongo L, Marmo F, Romano R, Iannotta M, Napolitano F, Belardo C, Marabese I, D'Aniello A, De Gregorio D, Rossi F, Piscitelli F, Lattanzi R, de Bartolomeis A, Usiello A, Di Marzo V, de Novellis V, Maione S (2015) Palmitoylethanolamide reduces pain-related behaviors and restores glutamatergic synapses homeostasis in the medial prefrontal cortex of neuropathic mice. *Mol Brain* **12**, 8:47.
- [17] D'Agostino G, Russo R, Avagliano C, Cristiano C, Meli R, Calignano A (2012) Palmitoylethanolamide protects against the amyloid- β 25-35-induced learning and memory impairment in mice, an experimental model of Alzheimer disease. *Neuropsychopharmacology* **37**, 1784-1792.
- [18] Scuderi C, Valenza M, Stecca C, Esposito G, Carratu' MR, Steardo L (2012) Palmitoylethanolamide exerts neuroprotective effects in mixed neuroglial cultures and organotypic hippocampal slices via peroxisome proliferator-activated receptor- α . *J Neuroinflammation* **9**, 49.
- [19] Scuderi C, Steardo L (2013) Neuroglial roots of neurodegenerative diseases: therapeutic potential of palmitoylethanolamide in models of Alzheimer's disease. *CNS Neurol Disord Drug Targets* **12**, 62-69.
- [20] Scuderi C, Stecca C, Valenza M, Ratano P, Bronzuoli MR, Bartoli S, Steardo L, Pompili E, Fumagalli L, Campolongo P, Steardo L (2014) Palmitoylethanolamide controls reactive gliosis and exerts neuroprotective functions in a rat model of Alzheimer's disease. *Cell Death Dis* **5**, e1419.
- [21] Paterniti I, Cordaro M, Campolo M, Siracusa R, Cornelius C, Navarra M, Cuzzocrea S, Esposito E (2014) Neuroprotection by association of palmitoylethanolamide with luteolin in experimental Alzheimer's disease models: the control of neuroinflammation. *CNS Neurol Disord Drug Targets* **13**, 1530-1541.
- [22] Cipriano M, Esposito G, Negro L, Capoccia E, Sarnelli G, Scuderi C, De Filippis D, Steardo L, Iuvone T (2015) Palmitoylethanolamide Regulates Production of Pro-Angiogenic Mediators in a Model of β Amyloid-Induced Astroglial In Vitro. *CNS Neurol Disord Drug Targets* **14**, 828-837.

- [23] Tomasini MC, Borelli AC, Beggiano S, Ferraro L, Cassano T, Tanganelli S, Antonelli T (2015) Differential Effects of Palmitoylethanolamide against Amyloid- β Induced Toxicity in Cortical Neuronal and Astrocytic Primary Cultures from Wild-Type and 3xTg-AD Mice. *J Alzheimers Dis* **46**, 407-421.
- [24] Calabrò RS, Naro A, De Luca R, Leonardi S, Russo M, Marra A, Bramanti P (2016) PEALut efficacy in mild cognitive impairment: evidence from a SPECT case study! *Aging Clin Exp Res* **28**, 1279-1282.
- [25] Antonelli T, Tomasini MC, Fournier J, Mazza R, Tanganelli S, Pirondi S, Fuxe K, Ferraro L (2008) Neurotensin receptor involvement in the rise of extracellular glutamate levels and apoptotic nerve cell death in primary cortical cultures after oxygen and glucose deprivation. *Cereb Cortex* **18**, 1748-1757.
- [26] Scuderi C, Esposito G, Blasio A, Valenza M, Arietti P, Steardo L Jr, Carnuccio R, De Filippis D, Petrosino S, Iuvone T, Di Marzo V, Steardo L (2011) Palmitoylethanolamide counteracts reactive astrogliosis induced by β -amyloid peptide. *J Cell Mol Med* **15**(12), 2664-74.
- [27] Repetto G, del Peso A, Zurita JL (2008) Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat Protoc* **3**, 1125-1131.
- [28] Ricci G, Volpi L, Pasquali L, Petrozzi L, Siciliano G (2009) Astrocyte-neuron interactions in neurological disorders. *J Biol Phys* **35**, 317-336.
- [29] Rossi D (2015) Astrocyte physiopathology: At the crossroads of intercellular networking, inflammation and cell death. *Prog Neurobiol* **130**, 86-120.
- [30] Rodríguez JJ, Butt AM, Gardenal E, Parpura V, Verkhratsky A (2016) Complex and differential glial responses in Alzheimer's disease and ageing. *Curr Alzheimer* **13**, 343-358.
- [31] Heneka MT, Rodríguez JJ, Verkhratsky A (2010) Neuroglia in neurodegeneration. *Brain Res Rev* **63**, 189-211.
- [32] Glantz LA, Gilmore JH, Lieberman JA, Jarskog LF (2006) Apoptotic mechanisms and the synaptic pathology of schizophrenia. *Schizophr Res* **81**, 47-63.
- [33] Falcão AS, Silva RF, Pancadas S, Fernandes A, Brito MA, Brites D (2007) Apoptosis and impairment of neurite network by short exposure of immature rat cortical neurons to unconjugated bilirubin increase with cell differentiation and are additionally enhanced by an inflammatory stimulus. *J Neurosci Res* **85**, 1229-1239.
- [34] Bisogno T, Martire A, Petrosino S, Popoli P, Di Marzo V (2008) Symptom-related changes of endocannabinoid and palmitoylethanolamide levels in brain areas of R6/2 mice, a transgenic model of Huntington's disease. *Neurochem Int* **52**, 307-313.

- [35] Tramutola A, Lanzillotta C, Perluigi M, Butterfield DA (2016) Oxidative stress, protein modification and Alzheimer disease. *Brain Res Bull* pii: S0361-9230(16)30129-0.
- [36] Jiang T, Sun Q, Chen S (2016) Oxidative stress: A major pathogenesis and potential therapeutic target of antioxidative agents in Parkinson's disease and Alzheimer's disease. *Prog Neurobiol* **147**, 1-19.
- [37] Abramov AY, Canevari L, Duchen MR (2004) Calcium signals induced by amyloid beta peptide and their consequences in neurons and astrocytes in culture. *Biochim Biophys Acta* **1742**, 81-87.
- [38] Angelova PR, Abramov AY (2014) Interaction of neurons and astrocytes underlies the mechanism of A β -induced neurotoxicity. *Biochem Soc Trans* **42**, 1286-1290.
- [39] Nestmann ER (2016). Safety of micronized palmitoylethanolamide (microPEA): lack of toxicity and genotoxic potential. *Food Sci Nutr* **5**, 292-309.

Figure legends

Figure 1. Effects of astrocytic A β ₄₂ pre-exposure (0.5 μ M, 24 hours), alone or in combination with PEA (0.1 μ M), on neuronal viability in mouse astrocyte-neuron co-cultures. PEA was added 1 hour before A β ₄₂ and maintained in contact with the cells during A β ₄₂ exposure. Cell viability was assessed by Neutral red assay and expressed as percentage of control values. Each histogram represents the mean + S.E.M (n=30-40). *p<0.05 significantly different from control, PEA and PEA + A β ₄₂ groups according to ANOVA followed by the Newman-Keuls test for multiple comparisons

Figure 2. Representative fluorescence photomicrographs of MAP-2 immunoreactivity showing the effects of astrocytic A β ₄₂ pre-exposure (0.5 μ M, 24 hours) on the morphology of primary cortical neurons obtained from mouse astrocyte-neuron co-cultures. Local neuronal network fragmentations are indicated by the white arrows. Neurons were stained with anti-MAP-2 antibody and observed in sample fields under fluorescent microscope (magnification 40x).

Figure 3. Effects of astrocytic A β ₄₂ pre-exposure (0.5 μ M, 24 hours), alone or in combination with PEA (0.1 μ M), on the number of MAP2 positive cells in mouse primary cortical neurons from astrocyte-neuron co-cultures. PEA was added 1 hour before A β ₄₂ and maintained in contact with the cells during A β ₄₂ exposure. Neurons were stained with anti-MAP2 antibody and observed under fluorescent microscope. Each histogram represents the mean \pm S.E.M (n=8/12). *p<0.05 significantly different from control, PEA and PEA + A β ₄₂ groups according to ANOVA followed by the Newman-Keuls test for multiple comparisons.

Figure 4. Effects of astrocytic A β ₄₂ pre-exposure (0.5 μ M, 24 hours), alone or in combination with PEA (0.1 μ M), on the number of aggregation/100 μ m in primary cortical neurons from astrocyte-neuron co-cultures. PEA was added 1 h before A β ₄₂ and maintained in contact with the cells during A β ₄₂ exposure. Neurons were stained with anti-MAP2 antibody and observed under fluorescent microscope. Each histogram represents the mean \pm S.E.M (n=8/12). ***p<0.001 significantly different from control, PEA and PEA + A β ₄₂ groups according to ANOVA followed by the Newman-Keuls test for multiple comparisons.

Figure 5. Representative fluorescence photomicrographs showing the effects of astrocytic A β ₄₂ pre-exposure (0.5 μ M, 24 hours) on neuronal apoptotic nuclei in mouse astrocyte-neuron co-cultures. The neurons were stained with Hoechst 33258 and observed in sampled fields under fluorescent microscope (magnification 20x).

Figure 6. Effects of astrocytic A β ₄₂ pre-exposure (0.5 μ M, 24 hours), alone or in combination with PEA (0.1 μ M), on the percentage of apoptotic nuclei in cultured cortical neurons obtained from astrocyte-neuron co-cultures. PEA was added 1 h before A β ₄₂ and maintained in contact with the cells during A β ₄₂ exposure. Neurons were stained with the specific DNA stain Hoechst 33258. * $p < 0.05$ significantly different from control, PEA and PEA + A β ₄₂ groups according to analysis of variance followed by the Newman-Keuls test for multiple comparisons.

Figure 1

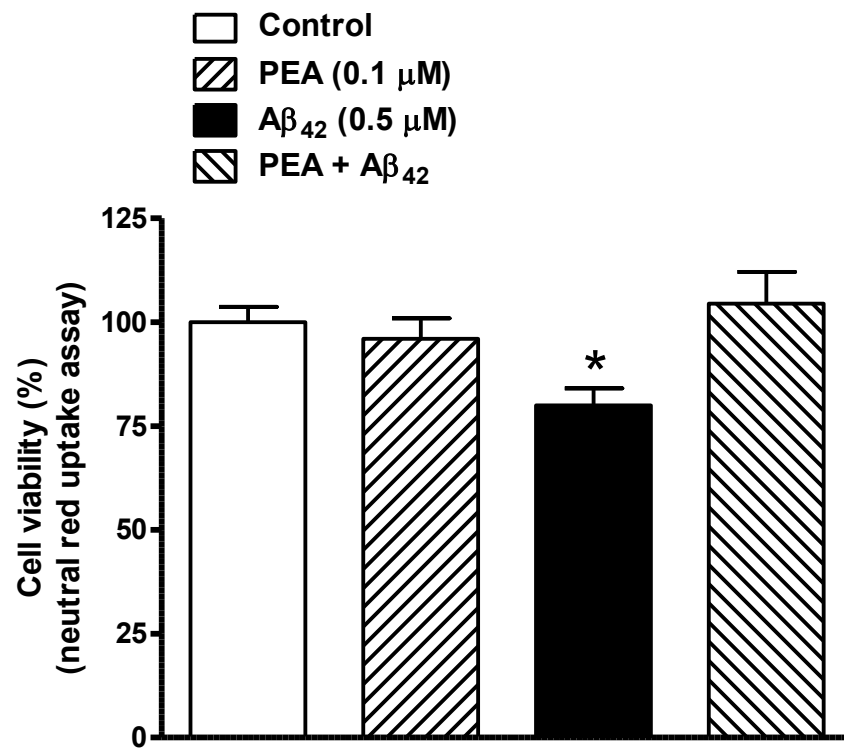


Figure 2

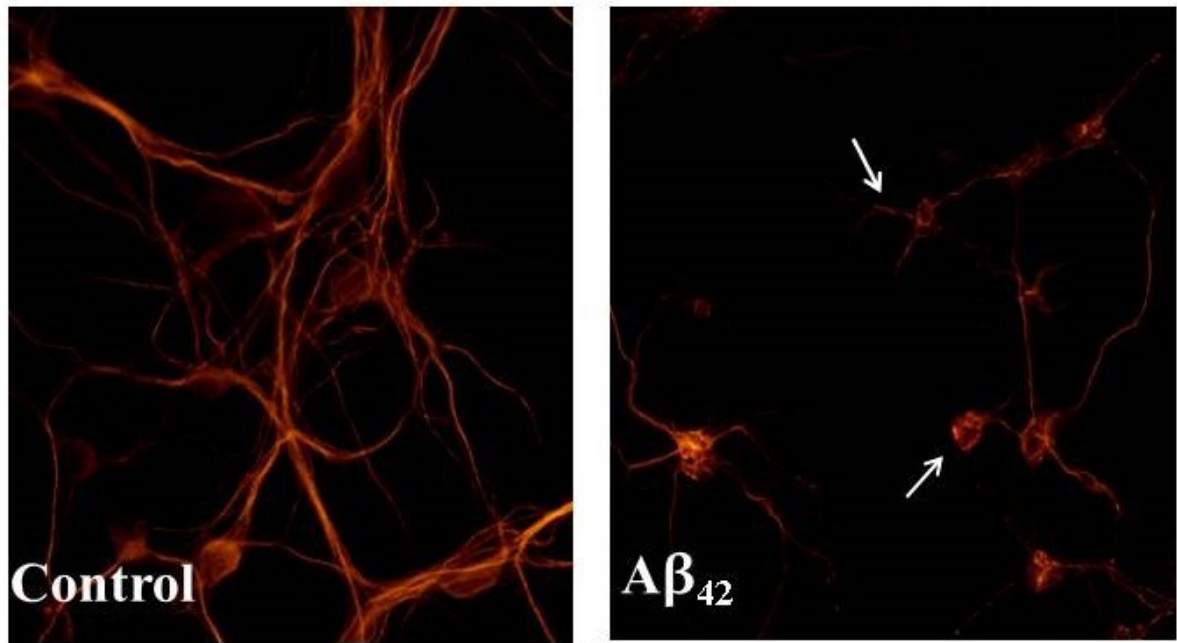


Figure 3

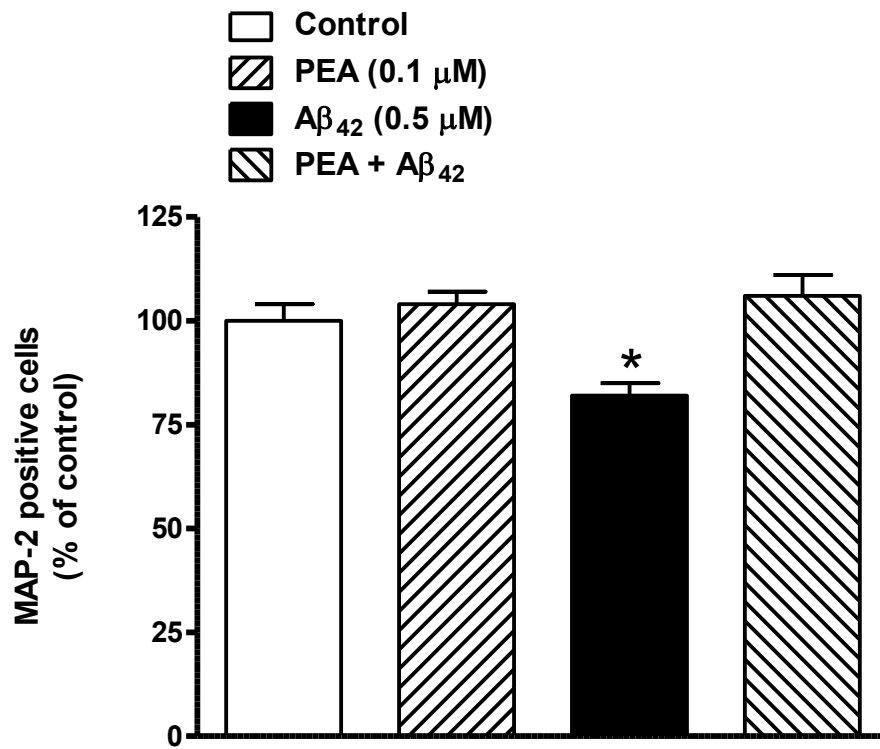


Figure 4

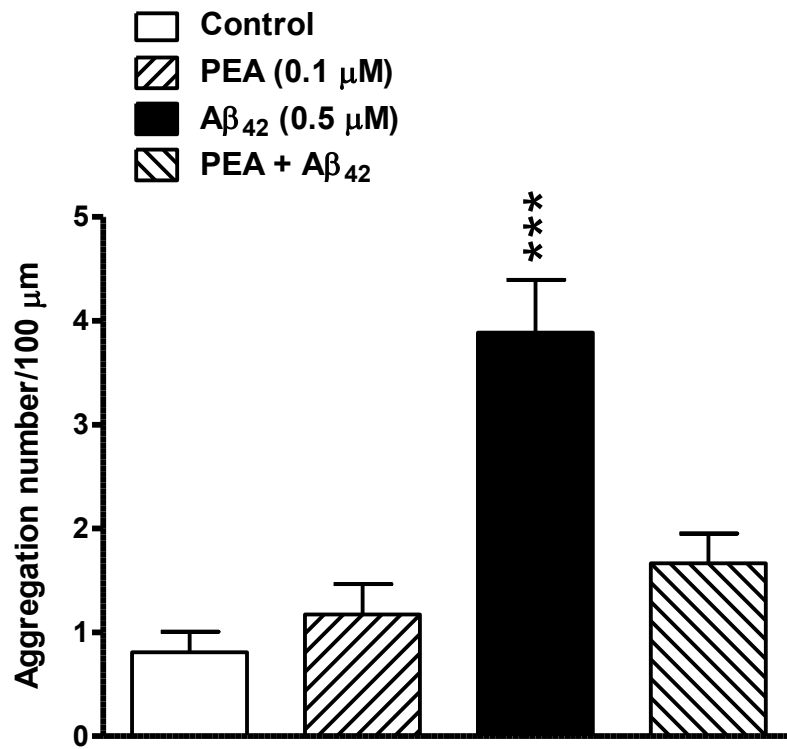


Figure 5

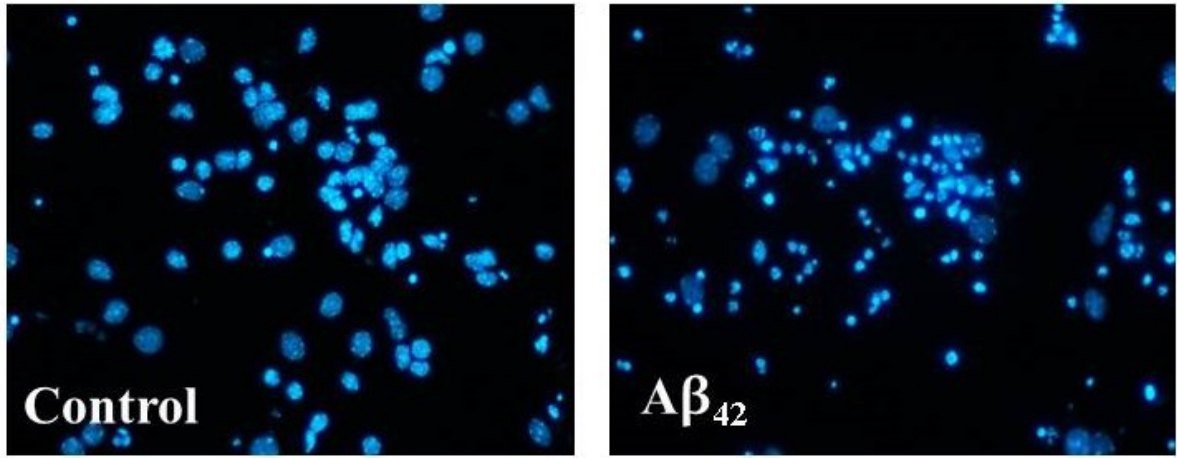


Figure 6

