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## WIN 55,212-2, agonist of cannabinoid receptors, prevents Amyloid $\beta$ 1-42 effects on astrocytes in primary culture.

--Manuscript Draft--

<b>Manuscript Number:</b>	PONE-D-14-56621R2
<b>Article Type:</b>	Research Article
<b>Full Title:</b>	WIN 55,212-2, agonist of cannabinoid receptors, prevents Amyloid $\beta$ 1-42 effects on astrocytes in primary culture.
<b>Short Title:</b>	WIN 55 prevents A $\beta$ 1-42 effects in cultured astrocytes
<b>Corresponding Author:</b>	Soraya L Valles University of Valencia VALENCIA, SPAIN
<b>Keywords:</b>	Amyloid $\beta$ 1-42, cannabinoids, inflammation, oxidative stress, cell death.
<b>Abstract:</b>	<p>Alzheimer's disease (AD), a neurodegenerative illness involving synaptic dysfunction with extracellular accumulation of A<math>\beta</math>1-42 toxic peptide, glial activation, inflammatory response and oxidative stress, can lead to neuronal death. Endogenous cannabinoid system is implicated in physiological and physiopathological events in central nervous system (CNS), and changes in this system are related to many human diseases, including AD. However, studies on the effects of cannabinoids on astrocytes functions are scarce. In primary cultured astrocytes we studied cellular viability using MTT assay. Inflammatory and oxidative stress mediators were determined by ELISA and Western-blot techniques both in the presence and absence of A<math>\beta</math>1-42 peptide. Effects of WIN 55,212-2 (a synthetic cannabinoid) on cell viability, inflammatory mediators and oxidative stress were also determined. A<math>\beta</math>1-42 diminished astrocytes viability, increased TNF-<math>\alpha</math> and IL-1<math>\beta</math> levels and p-65, COX-2 and iNOS protein expression while decreased PPAR-<math>\gamma</math> and antioxidant enzyme Cu/Zn SOD. WIN 55,212-2 pretreatment prevents all effects elicited by A<math>\beta</math>1-42. Furthermore, cannabinoid WIN 55,212-2 also increased cell viability and PPAR-<math>\gamma</math> expression in control astrocytes. In conclusion cannabinoid WIN 55,212-2 increases cell viability and anti-inflammatory response in cultured astrocytes. Moreover, WIN 55,212-2 increases expression of anti-oxidant Cu/Zn SOD and is able to prevent inflammation induced by A<math>\beta</math>1-42 in cultured astrocytes. Further studies would be needed to assess the possible beneficial effects of cannabinoids in Alzheimer's disease patients.</p>
<b>Order of Authors:</b>	<p>Diana Aguirre-Rueda</p> <p>Sol Guerra-Ojeda</p> <p>Martin Aldasoro</p> <p>Antonio Iradi</p> <p>Elena Obrador</p> <p>M<sup>a</sup> Dolores Mauricio</p> <p>Jose M<sup>a</sup> Vila</p> <p>Patricia Marchio</p> <p>Soraya L Valles</p>
<b>Opposed Reviewers:</b>	
<b>Response to Reviewers:</b>	<p>Here we indicate the response to reviewer and editor.</p> <p>Reviewer #1: I appreciate the efforts taken by the authors to adequately address the previous concerns with respect to this manuscript.</p> <p>Following are the two minor issues that I have.</p> <p>1. In the figure legend for Figure 7. modify the last line to match with the conclusion from this study (delete the part that WIN 55,212-2 prevents oxidative stress, since no</p>

	<p>direct measure of oxidative stress was done). Modifications in Figure 7 have been done to match with the conclusion.</p> <p>2. In revised manuscript, authors show that 10 <math>\mu</math>M A<math>\beta</math>1-42 increases MnSOD protein expression. In the discussion section (page 14), can the authors further speculate regarding the conclusion of this finding with respect to mitochondria and oxidative stress during Alzheimer's disease, since mitochondria are the major organelles producing superoxide radicals leading to oxidative stress. To adequately answer this, apart from astrocytes, which are the focus of this study, what is known regarding mitochondria and oxidative stress in Alzheimer's disease in other cells like neuronal cells? And does more MnSOD mean more H<sub>2</sub>O<sub>2</sub> production, that might be a mediator of the oxidative stress ? A paragraph about all suggestions indicated by the referee has been added in discussion.</p>
<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>
<p><b>Financial Disclosure</b></p> <p>Please describe all sources of funding that have supported your work. A complete funding statement should do the following:</p> <p>Include <b>grant numbers and the URLs</b> of any funder's website. Use the full name, not acronyms, of funding institutions, and use initials to identify authors who received the funding.</p> <p><b>Describe the role</b> of any sponsors or funders in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. If they had <u>no role</u> in any of the above, include this <u>sentence</u> at the end of your statement: "<i>The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.</i>"</p> <p>If the study was <b>unfunded</b>, provide a statement that clearly indicates this, for example: "<i>The author(s) received no specific funding for this work.</i>"</p> <p>* typeset</p>	<p>Generalitat Valenciana AP-073/09</p>
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<p>Additional data availability information:</p>	

February, 2015

Chief Editor  
Plos One

**Dear Editor**

Please find enclosed the manuscript **WIN 55,212-2, agonist of cannabinoid receptors, prevents Amyloid  $\beta_{1-42}$  effects on astrocytes in primary culture** by Aguirre-Rueda et al., which is intended for publication in Plos One after we correct again reviewer 1 suggestions.

Hoping the paper is now found suitable for publication in your Journal.

Yours sincerely,

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# **WIN 55,212-2, agonist of cannabinoid receptors, prevents Amyloid $\beta_{1-42}$ effects on astrocytes in primary culture.**

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**Abbreviations:** Alzheimer's disease (AD); amyloid beta<sub>40-1</sub> (A $\beta_{40-1}$ ); amyloid beta<sub>1-42</sub> (A $\beta_{1-42}$ ); peroxisome proliferator activated receptor gamma (PPAR- $\gamma$ ); WIN 55,212-2 (WIN); Cu/Zn superoxide dismutase (Cu/Zn SOD), Mn superoxide dismutase (Mn SOD), Central Nervous System (CNS).

**Keywords:** Amyloid  $\beta_{1-42}$ , cannabinoids, inflammation, oxidative stress, cell death.

# Abstract

Alzheimer's disease (AD), a neurodegenerative illness involving synaptic dysfunction with extracellular accumulation of  $A\beta_{1-42}$  toxic peptide, glial activation, inflammatory response and oxidative stress, can lead to neuronal death. Endogenous cannabinoid system is implicated in physiological and physiopathological events in central nervous system (CNS), and changes in this system are related to many human diseases, including AD. However, studies on the effects of cannabinoids on astrocytes functions are scarce. In primary cultured astrocytes we studied cellular viability using MTT assay. Inflammatory and oxidative stress mediators were determined by ELISA and Western-blot techniques both in the presence and absence of  $A\beta_{1-42}$  peptide. Effects of WIN 55,212-2 (a synthetic cannabinoid) on cell viability, inflammatory mediators and oxidative stress were also determined.  $A\beta_{1-42}$  diminished astrocytes viability, increased TNF- $\alpha$  and IL-1 $\beta$  levels and p-65, COX-2 and iNOS protein expression while decreased PPAR- $\gamma$  and antioxidant enzyme Cu/Zn SOD. WIN 55,212-2 pretreatment prevents all effects elicited by  $A\beta_{1-42}$ . Furthermore, cannabinoid WIN 55,212-2 also increased cell viability and PPAR- $\gamma$  expression in control astrocytes. In conclusion cannabinoid WIN 55,212-2 increases cell viability and anti-inflammatory response in cultured astrocytes. Moreover, WIN 55,212-2 increases expression of anti-oxidant Cu/Zn SOD and is able to prevent inflammation induced by  $A\beta_{1-42}$  in cultured astrocytes. Further studies would be needed to assess the possible beneficial effects of cannabinoids in Alzheimer's disease patients.



# Introduction

AD is a common neurodegenerative disease implicated in the aging process, affecting nearly 50% of people over 75 [1,2]. It involves neurofibrillary degeneration, extracellular accumulation of beta-amyloid peptide (A $\beta$ ) and synaptic dysfunction, resulting in neural cell death in the hippocampus and cerebral cortex, and in activation of glial cells [3,4]. A $\beta$  can interact with different cellular components producing Ca<sup>2+</sup> deregulation, oxidative stress and inflammation [5,6].

Astrocytes are specialized neural cells serving as a structural and metabolic support and trophic help to the brain [7]. Astrocytes also release cytokines and chemokines involved both in protective and toxic roles in neuroinflammatory processes [8]. However, released cytokines in neuroinflammation may induce deleterious effects on the viability and functionality of astrocytes [9]. Furthermore, in pathological situations such as hypoxia, cytokines induce activation of vascular endothelial cells thereby modulating inflammatory responses [10]. In AD, astrocytes are found around senile plaques producing phagocytosis, and cleaning up toxic compounds such as A $\beta$  [11]. Moreover, when stimulated with compounds such as genistein or estradiol, astrocytes block the release of pro-inflammatory mediators and induce the synthesis of anti-inflammatory proteins [12].

Endocannabinoids have been implicated in various physiopathological events in different organs, including the peripheral and CNS [13], and changes in the endocannabinoid system have been related to many human diseases, such as metabolic syndrome [14], neurodegeneration [15], inflammatory diseases [16], psychiatric disorders [17] and cancer [18]. The endocannabinoid signaling system is composed of anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) interacting with CB1 and CB2 cannabinoid receptors. Receptor signaling may involve mechanisms such as adenylyl

cyclase blockade or activation of mitogen-activated protein kinases or ceramide signaling [13].

Different authors have proposed cannabinoids as preventive treatment in AD [19] due to their anti-inflammatory and neuroprotective properties [16]. In this sense, cannabinoids prevented microglial activation and cognitive impairment in A $\beta$ -treated rats [19]. In mice exposed to A $\beta$ , cannabinoids also suppress neuroinflammation by inhibiting iNOS expression and interleukin-1 $\beta$  generation [20]. However, the effects of cannabinoids on astrocytes functions have been poorly investigated. Therefore, we investigated the role of WIN 55,212-2 (WIN) as a neuroprotective agent against lesions induced by A $\beta$ <sub>1-42</sub> on cultured astrocytes.

# Material and Methods

## Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Gibco Invitrogen Corporation, Barcelona, Spain). The oligomers A $\beta$  (40-1 and 1-42), were prepared following manufacture instructions (Sigma-Aldrich biotechnology). Briefly, the peptides were dissolved in H<sub>2</sub>O, and, for assembly the oligomers, preparations were heated for 24 h at 37°C. WIN and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St Louis, MO). Enzyme-linked immunosorbent assay (ELISA) kits for IL-1 $\beta$  and TNF- $\alpha$  from Pierce Biotechnology, Inc. (Rockford, USA). Western Blot Chemiluminescent Detection System (ECL) was from Amersham (Amersham Biosciences, Barcelona, Spain). Monoclonal anti-peroxisome proliferator-activated receptor antibody (PPAR- $\gamma$ ) (1:250) and polyclonal anti-cyclooxygenase-2 antibody (COX-2) (1:250) from Sigma Aldrich (Madrid, Spain). Monoclonal p65 antibody (p65) (1:250) from Santa Cruz Biotechnology (Madrid, Spain). Polyclonal anti-Cu/Zn superoxide dismutase antibody (Cu/Zn SOD) (1:250) from Assay Designs (Madrid, Spain). Monoclonal anti-tubuline (1:1000) from Cell Signaling (Beverly, MA, USA). All other reagents are analytical or culture grade purity.

## Primary culture of cortical astrocytes

All animals were handled according to the recommendations of the Bioethics Committee of the School of Medicine of the University of Valencia, Spain. Ethics committee specifically approved this study. Cortical astrocytes were isolated from rat fetuses of 21 days gestation. Fetuses were obtained by cesarean section and decapitated. Cerebral cortices were removed and triturated 10–15 times through a Pasteur pipette

with 10 ml DMEM. The cell suspension was filtered through nylon mesh with a pore size of 90  $\mu\text{m}$  and re-suspended in DMEM containing 20% fetal bovine serum (FBS), supplemented with L-glutamine (1%), HEPES (10 mM), fungizone (1%), and antibiotics (1%). Cells were plated on T75 culture flask and maintained in a humidified atmosphere of 5% CO<sub>2</sub>/95% air at 37°C during 15 days. After 4 days of culture, the FBS was maintained at 20% and after 1 week of culture, the FBS content was reduced to 10%, and the medium was changed twice a week. The purity of astrocytes was assessed by immunofluorescence using anti-gial fibrillary acidic protein (astrocyte marker; Sigma-Aldrich), anti-CD-68 (microglial marker; Serotec), anti-myelin basic protein (oligodendroglial marker; Sigma-Aldrich) and anti-microtubule-associated protein 2 (neuronal marker; Sigma-Aldrich). The astrocyte cultures were found to be at least 99% glial fibrillary acidic protein positive. No cells were found to express CD-68, myelin basic protein, or microtubule-associated protein-2.

## **Cell treatments**

Ten days after seeding, WIN (10  $\mu\text{M}$ ) was added to culture flasks. Twenty-four hours later, 10  $\mu\text{M}$  A $\beta$ <sub>1-42</sub> (toxic peptide) or A $\beta$ <sub>40-1</sub> (control peptide) (Sigma-Aldrich) were added to the flasks. A $\beta$ <sub>1-42</sub> concentration used in our study is in the range of toxic concentrations of the peptide [21,22]. Before incubation, the peptides were diluted in 100  $\mu\text{M}$  of phosphate-buffered saline (PBS) and incubated for 24 h at 37° C. Assays were performed 24 h after peptide addition.

## **MTT assay**

Cell viability was determined by MTT assay. The MTT assay is a well-established, widely used and easily reproducible method for the assessment of cell viability and cytotoxicity [23,24]. Astrocytes were plated in 96-well culture plate and incubated with

WIN during 24h. Subsequently, A $\beta$ <sub>40-1</sub> (control) and A $\beta$ <sub>1-42</sub> peptides were added to wells for another 24h. After cell treatments, the medium was removed and cells were incubated with red free medium and MTT solution [0.5 mg/ml, prepared in phosphate buffer saline (PBS) solution] for 4 h at 37°C. Finally, the medium was removed and formazan particles were dissolved in dimethyl sulfoxide (DMSO). Cell viability, defined as the relative amount of MTT reduction, was determined by spectrophotometry at 570 nm.

### **Cytokine determination, IL-1 and TNF $\alpha$**

Astrocytes were seeded as previously published [12]. At the time of assay, the red phenol medium was removed and replaced by PBS containing 1 mg/ml bovine serum albumin (BSA), either in the presence or absence of A $\beta$ <sub>1-42</sub> (10  $\mu$ M). IL-1 $\beta$  and TNF- $\alpha$  concentration (pg/ml) were ascertained using ELISA kits (Pierce Biotechnology, Inc.).

### **Western blot analysis**

Cultured cells were treated with lysis buffer and mechanically degraded to release the proteins. Protein concentration was determined using modified Lowry method [25]. Loading buffer (0.125 M Tris-HCl, pH 6.8, 2% SDS, 0.5% (v/v) 2-mercaptoethanol, 1% bromophenolblue and 19% glycerol) was added to protein sample and heated for 5 min at 95°C. Proteins were separated on SDS-PAGE gels and transferred to nitrocellulose membranes in a humid environment using a transfer buffer (25mM Tris, 190mM glycine, 20% methanol). Membranes were blocked with 5% milk in TBS (0.05% Tween-20) and incubated with primary antibodies overnight at 4°C. Membranes were washed 3 times with wash buffer TBS-T (TBS, 0.2% Tween-20) and incubated with a secondary anti-rabbit IgG or anti-mouse IgG (Cell Signalling Technologies Danvers, MA) antibody conjugated to the enzyme horseradish peroxidase (HRP) for 1 h. Membranes were washed three times and proteins were detected using the ECL method as specified by the

manufacturer. Autoradiography signals were assessed using digital image system ImageQuant LAS 4000 (GE Healthcare).

## **Statistical analyses**

Values are expressed as mean $\pm$ S.D. Differences between groups were assessed by one-way analysis of variance (ANOVA). Statistical significance was accepted at  $P \leq 0.05$ . Data sets in which  $F$  was significant were examined by a modified  $t$ -test.

## **Results**

### **Protective role of WIN on cell viability**

The role of WIN on cell viability was studied using MTT conversion assay. Figure 1A shows that incubation with WIN at different concentrations induced a significant increase in cell viability at 10  $\mu$ M. Consequently, that concentration was used in future experiments. Astrocytes previously incubated with 10  $\mu$ M  $A\beta_{1-42}$  for 24 h significantly decreased cell viability compared to control cells (Figure 1B). Furthermore, pretreating astrocytes during 48 h with WIN (10  $\mu$ M) prevented the decrease in cell viability induced by  $A\beta_{1-42}$  (WIN +  $A\beta$ ), conversely WIN (1, 2, 5 $\mu$ M) did not have any effect (Figure 1B).

### **WIN prevents IL-1 $\beta$ and TNF- $\alpha$ increase elicited by $A\beta_{1-42}$**

Cultured astrocytes were incubated with 10  $\mu$ M  $A\beta_{1-42}$  and proinflammatory mediators TNF- $\alpha$  and IL-1 $\beta$  were detected by ELISA.  $A\beta_{1-42}$  increased 4.5-fold IL-1 $\beta$  release (480.4 $\pm$ 150.3 pg/ml) compared with control (103.9 $\pm$ 82.9 pg/ml) (Figure 2A) and 2.4 fold TNF- $\alpha$  release (605.3 $\pm$ 103.4 pg/ml vs 210.5 $\pm$ 85.3 pg/ml in control group) (Figure 2B). Furthermore, WIN pre-treatment (10  $\mu$ M) prevented the increase in pro-inflammatory mediators induced by  $A\beta_{1-42}$  (Figure 2 A and B).

## **Effect of A $\beta$ <sub>1-42</sub> and WIN on p65 protein expression**

NF- $\kappa$ B, the pro-inflammatory transcription factor, is formed by different subunits. We measured p65 protein expression by western-blot. Incubation with A $\beta$ <sub>1-42</sub> increased p65 protein expression compared with control astrocytes (Figure 3), which was prevented by WIN pretreatment. ( $p < 0.05$  compared with A $\beta$ <sub>1-42</sub> treated astrocytes)

## **WIN prevents COX-2 and iNOS protein increase induced by A $\beta$ <sub>1-42</sub> peptide**

Incubation with A $\beta$ <sub>1-42</sub> significantly increased inflammatory proteins COX-2 (Figure 4A) and iNOS (Figure 4B) expressions compared to control. Furthermore, pretreating astrocytes with WIN prevented the effects produced by A $\beta$ <sub>1-42</sub>.

## **Effect of A $\beta$ <sub>1-42</sub> and WIN on PPAR- $\gamma$ protein expression**

Pro-inflammatory gene expression is downregulated by PPARs family [26]. We found that pretreatment with WIN (10  $\mu$ M) increased PPAR- $\gamma$  expression compared to control cells (Figure 5). Incubation with A $\beta$ <sub>1-42</sub> significantly decreased PPAR- $\gamma$  expression that was prevented by WIN pretreatment.

## **Effect of A $\beta$ <sub>1-42</sub> and WIN on Cu/Zn SOD and Mn SOD protein expression.**

Superoxide dismutase (SOD) is a key antioxidant enzyme. In our study, incubation with A $\beta$ <sub>1-42</sub> decreased Cu/Zn SOD expression in astrocytes in primary culture which was prevented by WIN pretreatment, evidencing that WIN could play a neuro-protective role against oxidative stress induced by A $\beta$ <sub>1-42</sub> peptide (Figure 6A). On the other hand, our results indicated that Mn SOD protein expression is increased in presence of A $\beta$ <sub>1-42</sub>.

Pretreatment with WIN did not prevent Mn SOD increase induced by A $\beta$ <sub>1-42</sub> (Figure 6B).



## Discussion

Oxidative stress and inflammation are the main mechanisms in the progression of various neurodegenerative diseases, including AD [27-30]. In our study, we determined different markers involved in inflammation and oxidative stress induced by the A $\beta$ <sub>1-42</sub> peptide in primary cultures of astrocytes, with the aim to assess the antioxidant and anti-inflammatory effects of cannabinoid WIN. We found that WIN significantly increased astrocytes viability compared to control cells. Furthermore, WIN prevented the decrease in astrocytes viability induced by A $\beta$ <sub>1-42</sub>.

It has been shown that cannabinoids preserve neurons from A $\beta$  exposure by activating MAP kinase cascade [31] and by anti-oxidative and anti-apoptotic effects [32]. Moreover, some studies demonstrated that cannabinoids protect glial cells from death [33,34]. Nevertheless, in cancer, where cells are highly proliferative and undifferentiated, treatment with cannabinoids can block cell proliferation in a dose dependent manner [35-38], demonstrating that the effects of cannabinoids on cell viability are probably dependent on cell type [39] and developmental stage [40].

Expression of CB1 [41] and CB2 [42] receptors in rat culture astrocytes have been published and also dual activation of both cannabinoid receptors by WIN 55,212-2 (the mixed non-selective CB1/CB2 agonist) in rat cortical astrocytes have been detected [41]. On the other hand, WIN confers its protective and anti-inflammatory effects against A $\beta$  injury through both CB1 and CB2 receptors [43]. Given that our results there is expression of both types of cannabinoid receptors (CB1 and CB2), it is likely that the effect of WIN observed in our study is due to the interaction with both types of receptors, consistent with published results by Fakhfouri and cols [44].

We found that WIN prevented the increase of inflammatory mediators IL-1 $\beta$ , TNF- $\alpha$ , NF- $\kappa$ B, iNOS and COX-2, as well as the decrease of the anti-inflammatory mediator

PPAR- $\gamma$  induced by A $\beta_{1-42}$  in astrocytes in primary culture. The inflammatory process is a characteristic mechanism in the development of AD, and pro-inflammatory agents are involved in the progression of cell damage [45,46,47,12]. Moreover, it is known that astrocytes participate in the inflammatory process induced by A $\beta_{1-42}$  [27,28,48]. Initially, inflammation is beneficial since it produces pro-inflammatory substances involved in tissue protection, limiting the survival and proliferation of cells exposed to toxic agents, such as A $\beta_{1-42}$  [49,50]. However, sustained inflammatory response could lead to neurotoxic damage or cell death [12,51,8]. NF- $\kappa$ B proteins are up-regulated in inflammation conditions such as astroglial activation induced by A $\beta_{1-42}$  oligomers [52]. In this regard, we found an increase in NF- $\kappa$ B/p-65 expression in astrocytes after addition of A $\beta_{1-42}$  that was prevented by WIN pretreatment. Valles and collaborators [53] found that the cytokine-receptor complex is able to bind to cytokines and other proteins of the extracellular matrix, producing inflammatory signals which could be important in pathologies such as Alzheimer's disease [53,54]. In agreement with our results, different authors have reported that cannabinoids mitigate neural cell activation in the neuroinflammatory response induced by A $\beta_{1-42}$ , reducing the levels of pro-inflammatory molecules such as IL-1 $\beta$ , TNF- $\alpha$ , COX-2 and iNOS [55,56,57]. Likewise, the activation of cannabinoid receptors diminishes the release of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in microglial cells [58,59,60] as well as COX-2 and iNOS [61]. Studies conducted in rats pretreated with the A $\beta$  peptide found that WIN prevented cognitive impairment, glial activation and neuronal loss [19,62,63], and also reduced COX-2, iNOS and TNF- $\alpha$  levels [63,64].

Kainu et al. [65] demonstrated for the first time the presence of mRNA and protein PPAR- $\gamma$  in CNS cells. Subsequent studies have detected PPAR- $\gamma$  expression in microglial and astrocytic cells [66,12]. PPAR- $\gamma$  agonists protect against A $\beta$ -induced

inflammatory and neuronal damage [67,68], thus making neurons and astrocytes potential therapeutic targets for PPAR- $\gamma$  ligands [69,12]. Astrocytes also express the largest levels of PPAR- $\gamma$  in the neural tissues [70,71]. As other authors [72,73,74], we found a decreased expression of PPAR- $\gamma$  in astrocytes treated with A $\beta_{1-42}$ . Esposito et al. [56] showed in neurons that cannabinoids may act as neuro-protective agents by PPAR- $\gamma$  activation. In this study, we demonstrate an increase in this protein expression in astrocytic cells previously incubated with WIN. Furthermore, we found for the first time that WIN prevents PPAR- $\gamma$  expression decrease induced by A $\beta_{1-42}$  peptide in astrocytes in primary culture. There is strong evidence to suggest that some cannabinoids can act on PPARs through either direct or indirect pathways. In order to directly act on nuclear transcriptional factors PPARs, exogenous cannabinoids need to pass through plasma membrane and be transported into nucleus which may involve certain membrane and intracellular transporters. However, we still cannot rule out that cannabinoids effects could be indirect through the binding of other cellular targets which in turn induces PPARs activation [75]. In fact, WIN attenuates amyloid-beta-induced neuroinflammation in rats through activation of cannabinoid receptors and PPAR- $\gamma$  pathway [44].

Different authors have demonstrated the role of oxidative stress in AD [76-79]. The cumulative damage caused by free radicals induces alterations in the activity or expression of antioxidant enzymes like catalase or SOD. These enzymes were found to be decreased in both CNS and peripheral tissues of AD patients [80,81]. In this sense, we demonstrate that Cu/Zn SOD is decreased in astrocytes treated with A $\beta_{1-42}$ . Our results are consistent with those reported by other authors, highlighting the role of oxidative stress in the development of AD [82]. New substances are under research to reduce damage caused by oxidative stress in this disease. Widely distributed in the body,

cannabinoids receptors were discovered few decades ago and are still under research [57]. Few studies address the effect of cannabinoids on oxidative stress. For instance, cannabinoids were found to prevent or antagonize oxidative stress toxicity in cortical neurons in cultures [83,84], and in lymphoblastic cells [85]. Studies with PC12 cells exposed to A $\beta_{1-42}$  peptide demonstrated that cannabinoids reduced reactive oxygen species production and membrane lipid oxidation [86,32]. Our results provide evidence that A $\beta_{1-42}$  decreases Cu/Zn SOD expression in astrocytes in primary culture, and pretreatment with WIN increases Cu/Zn SOD expression, preventing the decrease caused by A $\beta_{1-42}$ . These findings indicate that cannabinoids could act as a protective agent against oxidative stress caused by A $\beta_{1-42}$ . In Figure 7 the set of results are summarized. However our results indicate that A $\beta_{1-42}$  elevated Mn SOD protein expression, increasing mitochondrial biogenesis mechanism, such as we previously published [74]. Pretreatment with WIN did not prevent Mn SOD overexpression induced by A $\beta_{1-42}$ . Mn SOD plays a role in the adaptive response which protects brain cells from damage, as in the case of AD. In fact, Mn SOD preserves neurons against oxidative stress [87] and protects developing neurons from  $\beta$ -amyloid toxicity [88]. This enzyme catalyzes the conversion of superoxide radicals to molecular oxygen and H<sub>2</sub>O<sub>2</sub>, whereas glutathione peroxidase, peroxiredoxin reductase and catalase neutralize H<sub>2</sub>O<sub>2</sub>. Overexpression of Mn SOD induces cognitive recovery and reduces A $\beta$  levels in AD animal models [89]. Furthermore, Mn SOD deficiency increases  $\beta$ -amyloid levels and amyloid plaque burden, promoting the development of behavioural disturbances [90].

Preclinical data suggest a beneficial role of some cannabinoids for treatment of different diseases. Dronabidol, an oil-based solution of  $\Delta$ 9-THC, is used as anti-emetic and appetite stimulant [91].  $\Delta$ 9-THC also decreases agitation present in the advanced stage of AD [92]. In 2003, the FDA granted the patent for cannabinoids as antioxidants

and neuro-protectants (U.S. Department of Health and Human Services). Despite these promising preliminary results, the clinical utility of cannabinoids in AD is still to be determined [93].

## Conclusions

Taken together, our findings show that cannabinoid WIN increases cell viability and anti-inflammatory response in astrocytes in primary culture and prevents cell death induced by  $A\beta_{1-42}$ . Furthermore, WIN increases expression of anti-oxidant Cu/Zn SOD and is able to prevent inflammation induced by  $A\beta_{1-42}$  in astrocytes. In this sense, clinical studies are needed to evaluate the neuro-protective effects of cannabinoids in Alzheimer's disease.

## Conflict of Interest Statement:

The authors declare that there are no conflicts of interest.

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## Figure Legends

**Figure 1. Astrocytes viability.** (A) Astrocytes viability induced by WIN. Concentration-dependent viability of WIN (1, 2, 5, 10  $\mu\text{M}$ ) was determined by MTT assay for 24 h. Data are means  $\pm$  SD for 4 independent experiments.  $*p < 0.04$  comparing WIN vs control cells. (B) Astrocytes viability in cells treated during 24 h with 10  $\mu\text{M}$   $\text{A}\beta_{40-1}$  (control peptide, C), 10  $\mu\text{M}$   $\text{A}\beta_{1-42}$  ( $\text{A}\beta$ ) and WIN (1, 2, 5, 10  $\mu\text{M}$ ) + 10  $\mu\text{M}$   $\text{A}\beta_{1-42}$  (WIN +  $\text{A}\beta$ ). Data are means  $\pm$  SD of 3 independent experiments.  $*p < 0.05$  vs control cells.

**Figure 2. IL-1 $\beta$  and TNF- $\alpha$  secretion.** WIN prevents the increase of IL-1 $\beta$  and TNF- $\alpha$  secretion caused by  $\text{A}\beta_{1-42}$  in astrocytes. Cells were incubated with 10  $\mu\text{M}$   $\text{A}\beta_{40-1}$  (control peptide, C), 10  $\mu\text{M}$   $\text{A}\beta_{1-42}$  ( $\text{A}\beta$ ), 10  $\mu\text{M}$  WIN + 10  $\mu\text{M}$  control peptide (WIN) and 10  $\mu\text{M}$  WIN + 10  $\mu\text{M}$   $\text{A}\beta_{1-42}$  (WIN +  $\text{A}\beta$ ). Cell culture supernatants were harvested, and IL-1 $\beta$  (panel A) and TNF- $\alpha$  (panel B) secretion were determined by ELISA. Values are means  $\pm$  SD of replicate experiments from 4 independent astrocytes cultures.  $*p < 0.05$  vs control astrocytes.  $\#p < 0.05$  vs  $\text{A}\beta_{1-42}$  treated cells.

**Figure 3. p65 protein expression.** WIN 55, 212-2 prevents p65 expression induced by  $\text{A}\beta_{1-42}$  in astrocytes in primary culture. p65 and  $\alpha$ -tubulin expressions were determined by Western-blot in astrocytes treated for 24 h with 10  $\mu\text{M}$   $\text{A}\beta_{40-1}$  (control peptide, C), 10  $\mu\text{M}$   $\text{A}\beta_{1-42}$  ( $\text{A}\beta$ ), 10  $\mu\text{M}$  WIN + 10  $\mu\text{M}$  control peptide (WIN) and 10  $\mu\text{M}$  WIN + 10  $\mu\text{M}$   $\text{A}\beta_{1-42}$  (WIN +  $\text{A}\beta$ ). A representative immunoblot of each protein is shown and tubulin was used as control amount of protein. Data are means  $\pm$  SD of 5 independent experiments.  $*p < 0.05$  vs control cells.  $\#p < 0.05$  vs  $\text{A}\beta_{1-42}$ .

**Figure 4. COX-2 and iNOS protein expression.** WIN prevents COX-2 and iNOS expression induced by A $\beta$ <sub>1-42</sub>. COX-2 (panel A), iNOS (panel B) and  $\alpha$ -tubulin expressions were determined by Western-blot in astrocytes treated for 24 h with 10  $\mu$ M A $\beta$ <sub>40-1</sub> (control peptide, C), 10  $\mu$ M A $\beta$ <sub>1-42</sub> (A $\beta$ ), 10  $\mu$ M WIN + 10  $\mu$ M control peptide (WIN) and 10  $\mu$ M WIN + 10  $\mu$ M A $\beta$ <sub>1-42</sub> (WIN + A $\beta$ ). A representative immunoblot of each protein is shown and tubulin was used as control amount of protein. Data are means  $\pm$  SD of 6 independent experiments. \* $p$ <0.05 vs control cells. # $p$ <0.05 vs A $\beta$ <sub>1-42</sub>.

**Figure 5. PPAR- $\gamma$  protein expression.** WIN induces PPAR- $\gamma$  expression in astrocytes in primary culture treated with A $\beta$ <sub>1-42</sub>. PPAR- $\gamma$  and  $\alpha$ -tubulin expressions were determined by Western-blot in astrocytes treated for 24 h with 10  $\mu$ M A $\beta$ <sub>40-1</sub> (control peptide, C), 10  $\mu$ M A $\beta$ <sub>1-42</sub> (A $\beta$ ), 10  $\mu$ M WIN + 10  $\mu$ M control peptide (WIN) and 10  $\mu$ M WIN + 10  $\mu$ M A $\beta$ <sub>1-42</sub> (WIN + A $\beta$ ). A representative immunoblot of each protein is shown and tubulin was used as control amount of protein. Data are means  $\pm$  SD of 4 independent experiments. \* $p$ <0.05 vs control cells. # $p$ <0.05 vs A $\beta$ <sub>1-42</sub>.

**Figure 6. Cu/Zn-SOD and Mn-SOD protein expressions.** WIN prevents Cu/Zn-SOD expression decrease in astrocytes in primary culture treated with A $\beta$ <sub>1-42</sub>. Cu/Zn-SOD, Mn-SOD and  $\alpha$ -tubulin expressions were determined by Western-blot in astrocytes treated for 24 h with 10  $\mu$ M A $\beta$ <sub>40-1</sub> (control peptide, C), 10  $\mu$ M A $\beta$ <sub>1-42</sub> (A $\beta$ ), 10  $\mu$ M WIN + 10  $\mu$ M control peptide (WIN) and 10  $\mu$ M WIN + 10  $\mu$ M A $\beta$ <sub>1-42</sub> (WIN + A $\beta$ ). A representative immunoblot of each protein is shown and tubulin was used as control amount of protein. Data are means  $\pm$  SD of 4 independent experiments. \* $p$ <0.05 vs control cells. # $p$ <0.05 vs A $\beta$ <sub>1-42</sub>.

**Figure 7. Preventive function of cannabinoid WIN on A $\beta$ <sub>1-42</sub>-induced toxic effects in astrocytes in primary culture.** Cannabinoid WIN 55,212-2 increases cell viability and anti-inflammatory response in cultured astrocytes and prevents inflammatory effects induced by A $\beta$ <sub>1-42</sub>.

Figure 1  
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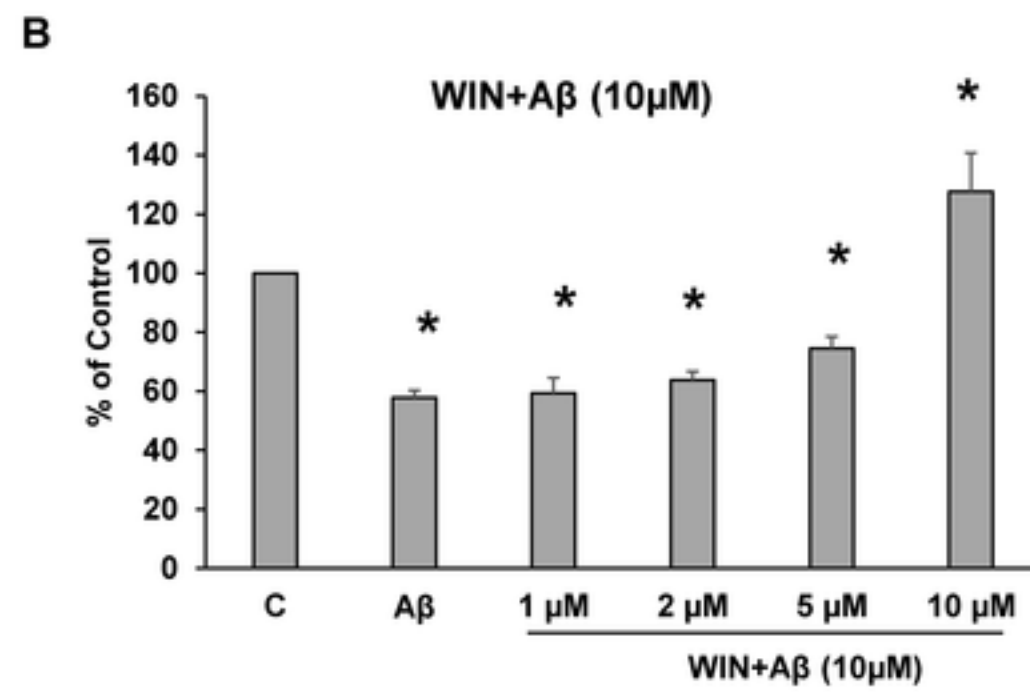
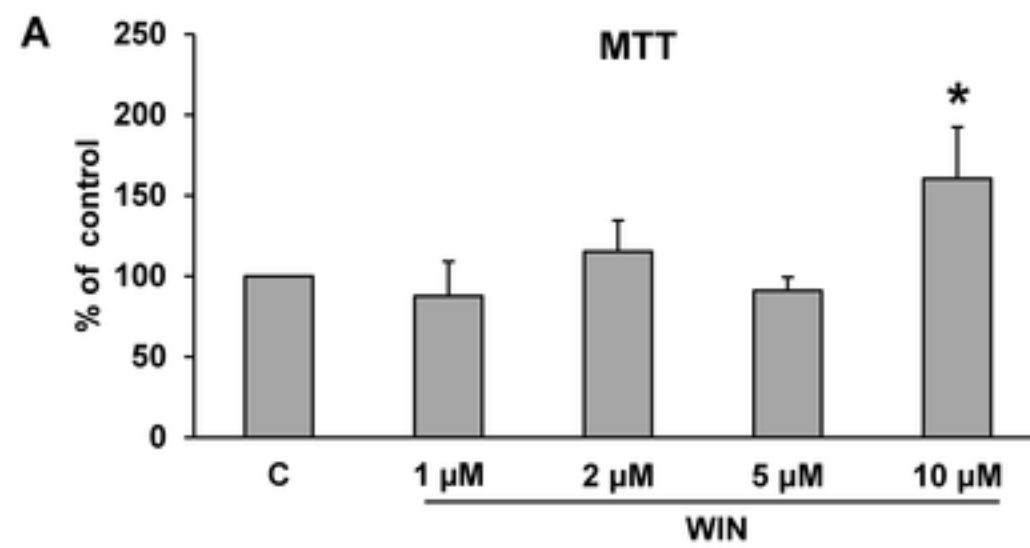


Figure 2

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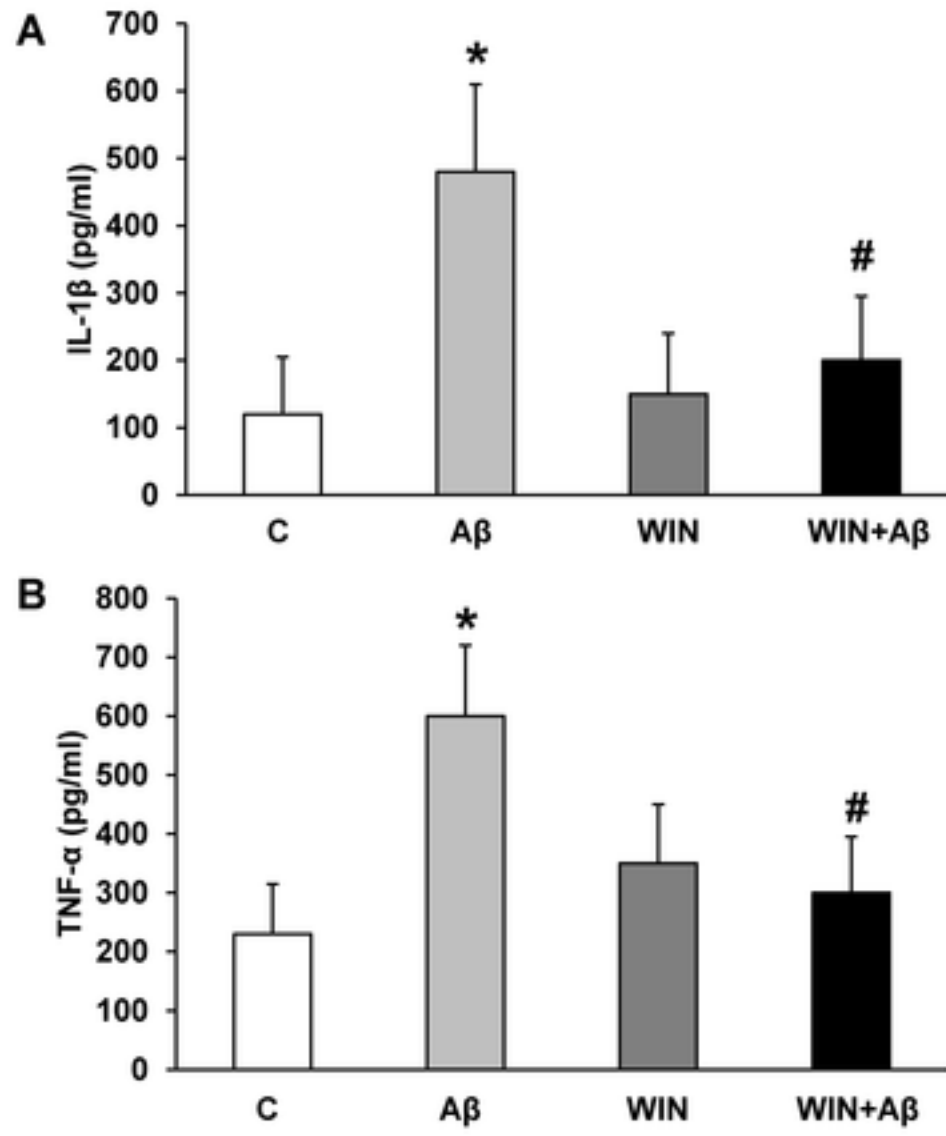


Figure 3  
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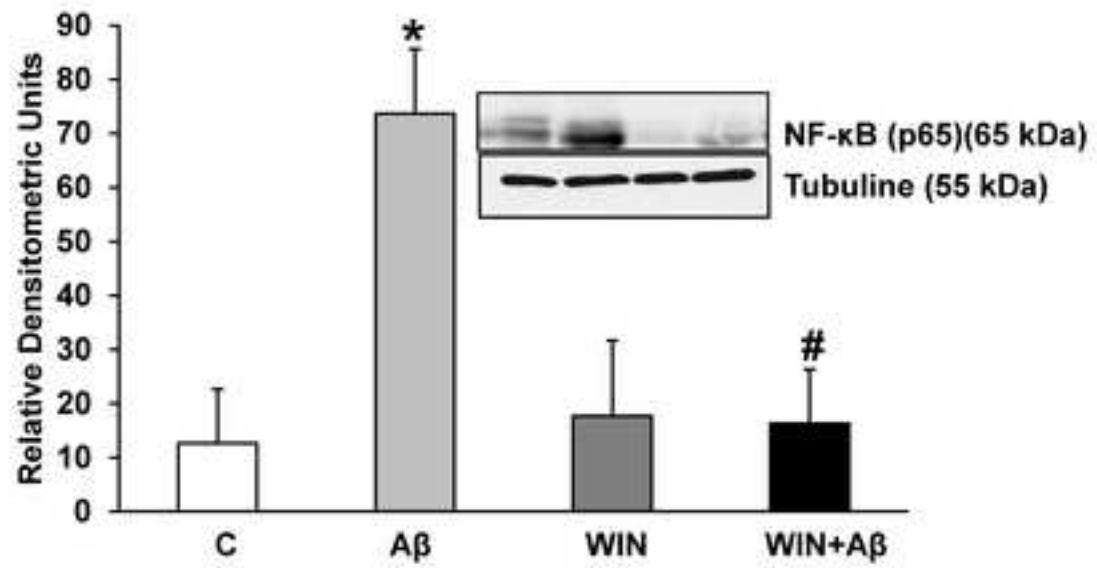


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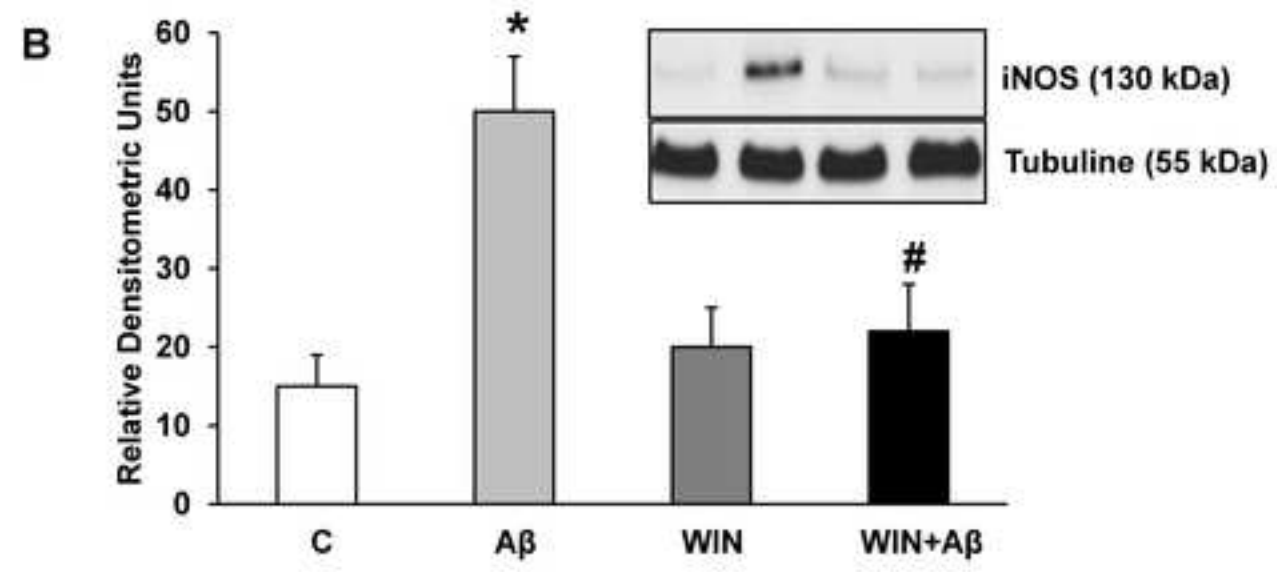
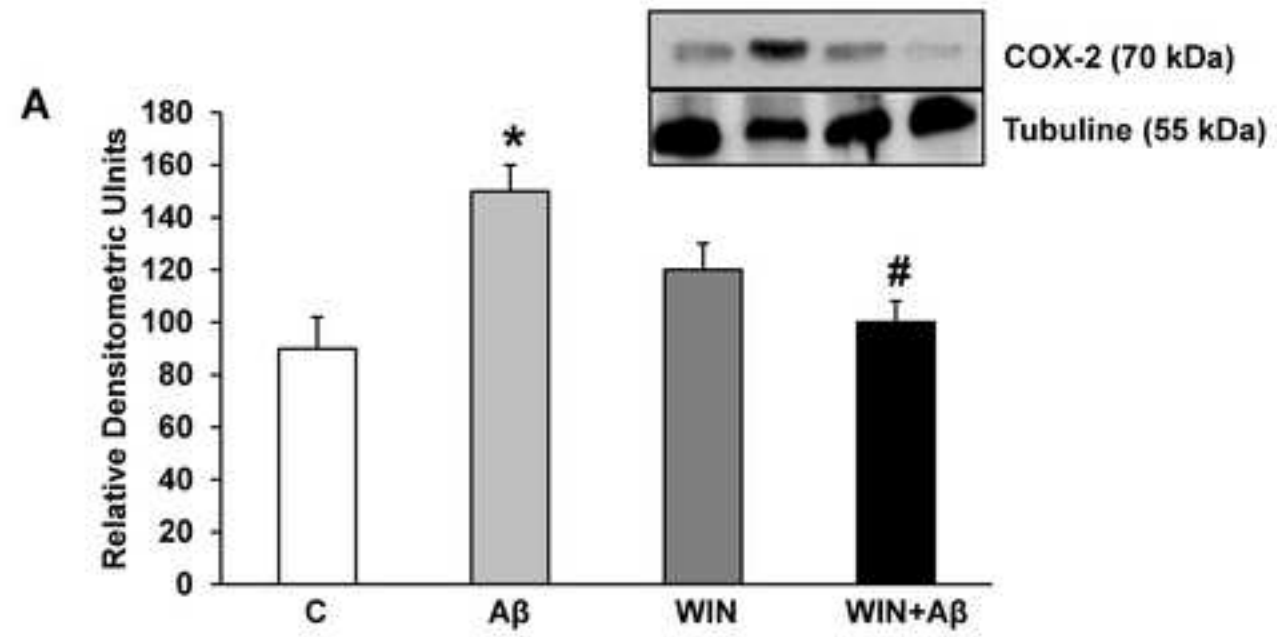


Figure 5  
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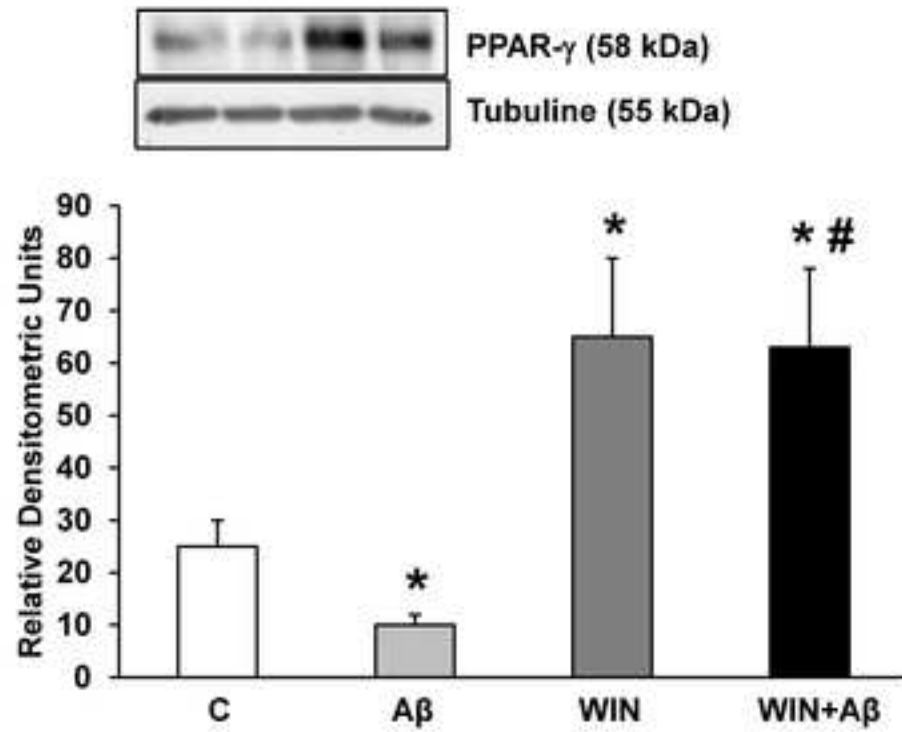




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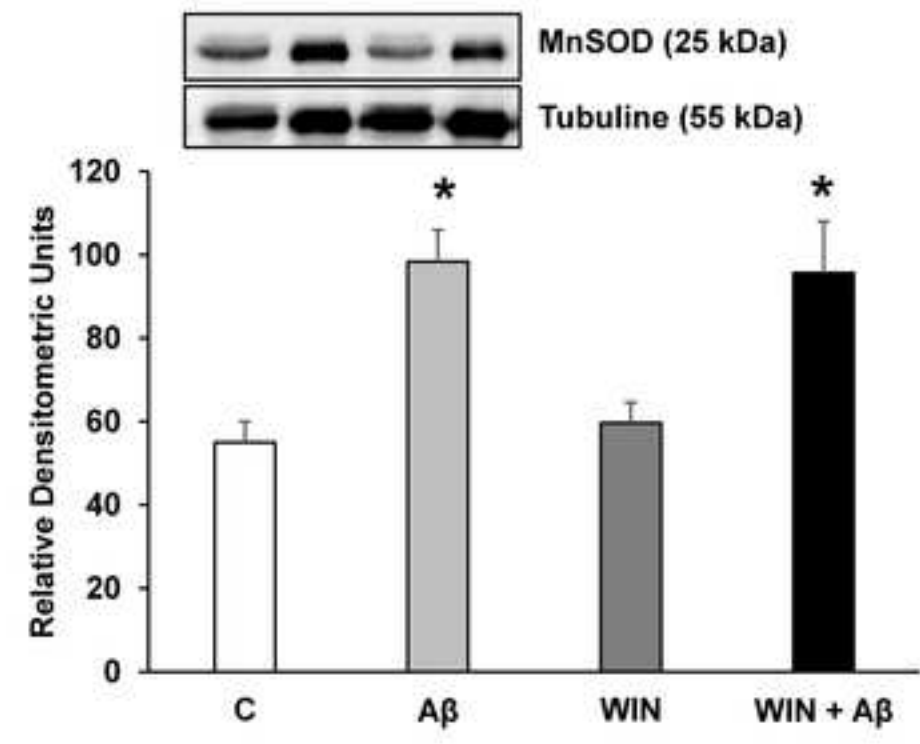
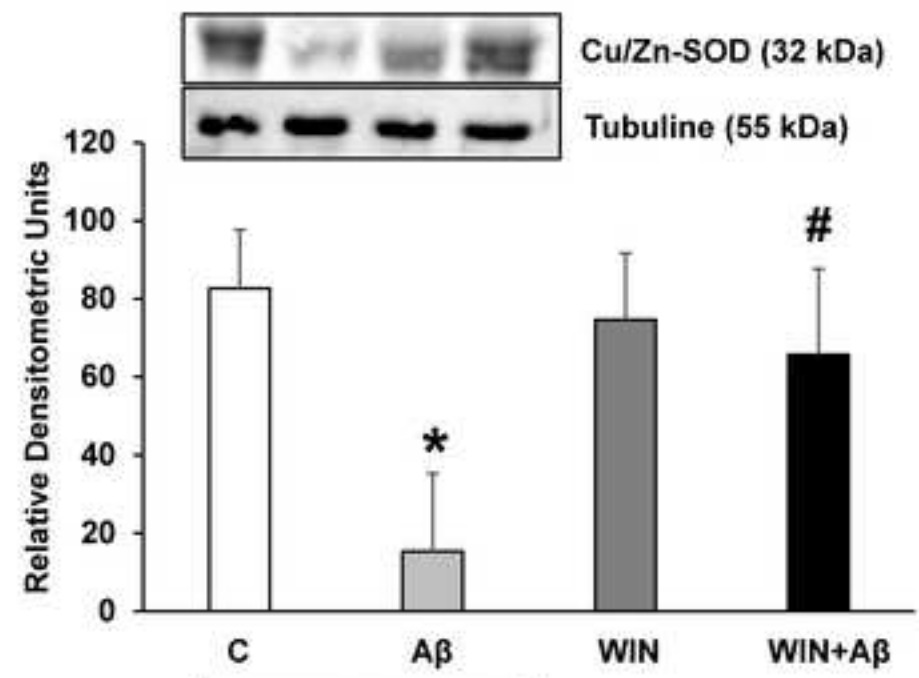
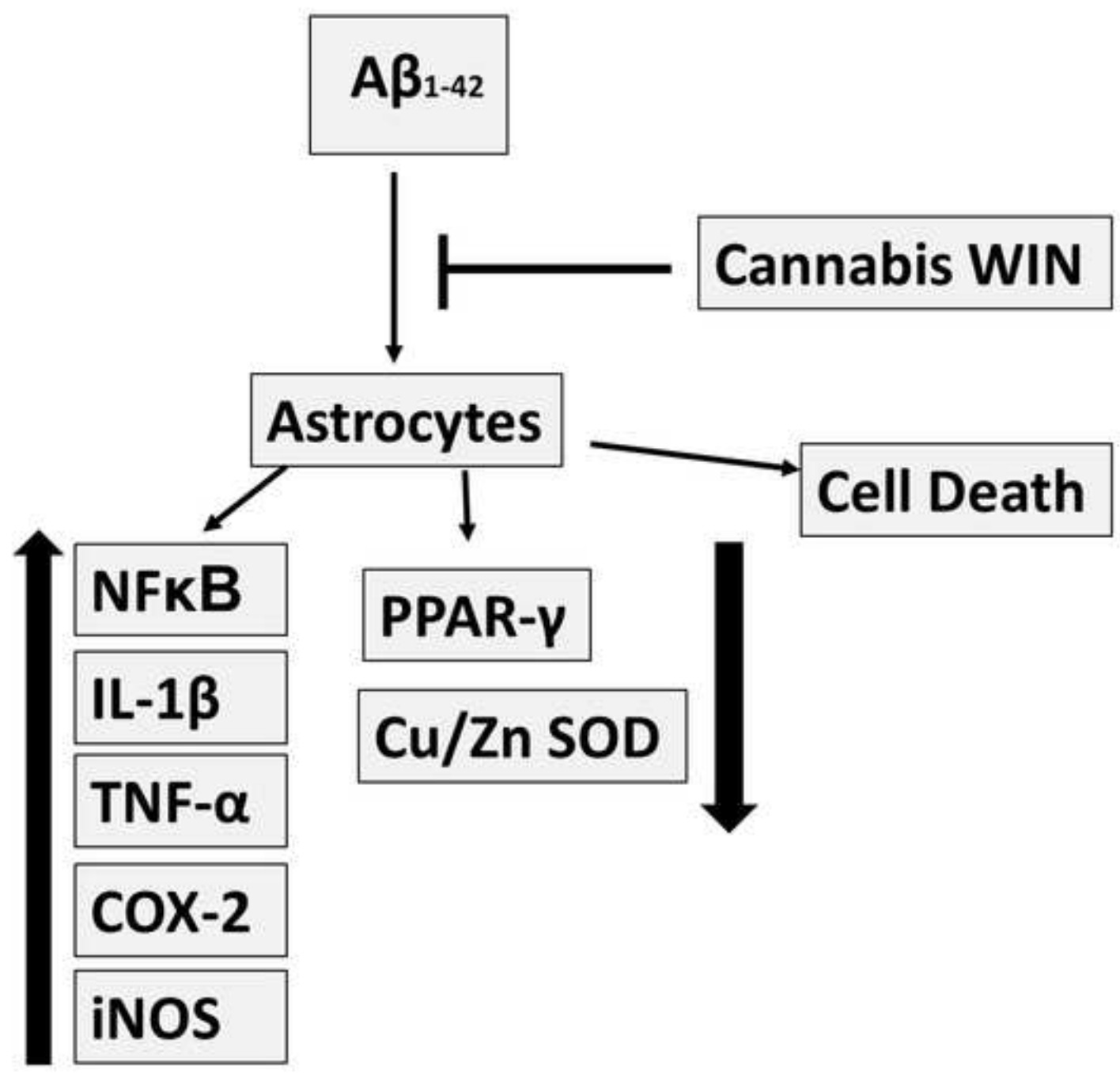


Figure 7  
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# **WIN 55,212-2, agonist of cannabinoid receptors, prevents Amyloid $\beta_{1-42}$ effects on astrocytes in primary culture.**

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**Abbreviations:** Alzheimer's disease (AD); amyloid beta<sub>40-1</sub> (A $\beta_{40-1}$ ); amyloid beta<sub>1-42</sub> (A $\beta_{1-42}$ ); peroxisome proliferator activated receptor gamma (PPAR- $\gamma$ ); WIN 55,212-2 (WIN); Cu/Zn superoxide dismutase (Cu/Zn SOD), Mn superoxide dismutase (Mn SOD), Central Nervous System (CNS).

**Keywords:** Amyloid  $\beta_{1-42}$ , cannabinoids, inflammation, oxidative stress, cell death.

# Abstract

Alzheimer's disease (AD), a neurodegenerative illness involving synaptic dysfunction with extracellular accumulation of  $A\beta_{1-42}$  toxic peptide, glial activation, inflammatory response and oxidative stress, can lead to neuronal death. Endogenous cannabinoid system is implicated in physiological and physiopathological events in central nervous system (CNS), and changes in this system are related to many human diseases, including AD. However, studies on the effects of cannabinoids on astrocytes functions are scarce. In primary cultured astrocytes we studied cellular viability using MTT assay. Inflammatory and oxidative stress mediators were determined by ELISA and Western-blot techniques both in the presence and absence of  $A\beta_{1-42}$  peptide. Effects of WIN 55,212-2 (a synthetic cannabinoid) on cell viability, inflammatory mediators and oxidative stress were also determined.  $A\beta_{1-42}$  diminished astrocytes viability, increased TNF- $\alpha$  and IL-1 $\beta$  levels and p-65, COX-2 and iNOS protein expression while decreased PPAR- $\gamma$  and antioxidant enzyme Cu/Zn SOD. WIN 55,212-2 pretreatment prevents all effects elicited by  $A\beta_{1-42}$ . Furthermore, cannabinoid WIN 55,212-2 also increased cell viability and PPAR- $\gamma$  expression in control astrocytes. In conclusion cannabinoid WIN 55,212-2 increases cell viability and anti-inflammatory response in cultured astrocytes. Moreover, WIN 55,212-2 increases expression of anti-oxidant Cu/Zn SOD and is able to prevent inflammation induced by  $A\beta_{1-42}$  in cultured astrocytes. Further studies would be needed to assess the possible beneficial effects of cannabinoids in Alzheimer's disease patients.

# Introduction

AD is a common neurodegenerative disease implicated in the aging process, affecting nearly 50% of people over 75 [1,2]. It involves neurofibrillary degeneration, extracellular accumulation of beta-amyloid peptide (A $\beta$ ) and synaptic dysfunction, resulting in neural cell death in the hippocampus and cerebral cortex, and in activation of glial cells [3,4]. A $\beta$  can interact with different cellular components producing Ca<sup>2+</sup> deregulation, oxidative stress and inflammation [5,6].

Astrocytes are specialized neural cells serving as a structural and metabolic support and trophic help to the brain [7]. Astrocytes also release cytokines and chemokines involved both in protective and toxic roles in neuroinflammatory processes [8]. However, released cytokines in neuroinflammation may induce deleterious effects on the viability and functionality of astrocytes [9]. Furthermore, in pathological situations such as hypoxia, cytokines induce activation of vascular endothelial cells thereby modulating inflammatory responses [10]. In AD, astrocytes are found around senile plaques producing phagocytosis, and cleaning up toxic compounds such as A $\beta$  [11]. Moreover, when stimulated with compounds such as genistein or estradiol, astrocytes block the release of pro-inflammatory mediators and induce the synthesis of anti-inflammatory proteins [12].

Endocannabinoids have been implicated in various physiopathological events in different organs, including the peripheral and CNS [13], and changes in the endocannabinoid system have been related to many human diseases, such as metabolic syndrome [14], neurodegeneration [15], inflammatory diseases [16], psychiatric disorders [17] and cancer [18]. The endocannabinoid signaling system is composed of anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) interacting with CB1 and CB2 cannabinoid receptors. Receptor signaling may involve mechanisms such as adenylyl

cyclase blockade or activation of mitogen-activated protein kinases or ceramide signaling [13].

Different authors have proposed cannabinoids as preventive treatment in AD [19] due to their anti-inflammatory and neuroprotective properties [16]. In this sense, cannabinoids prevented microglial activation and cognitive impairment in A $\beta$ -treated rats [19]. In mice exposed to A $\beta$ , cannabinoids also suppress neuroinflammation by inhibiting iNOS expression and interleukin-1 $\beta$  generation [20]. However, the effects of cannabinoids on astrocytes functions have been poorly investigated. Therefore, we investigated the role of WIN 55,212-2 (WIN) as a neuroprotective agent against lesions induced by A $\beta$ <sub>1-42</sub> on cultured astrocytes.

# Material and Methods

## Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Gibco Invitrogen Corporation, Barcelona, Spain). The oligomers A $\beta$  (40-1 and 1-42), were prepared following manufacture instructions (Sigma-Aldrich biotechnology). Briefly, the peptides were dissolved in H<sub>2</sub>O, and, for assembly the oligomers, preparations were heated for 24 h at 37°C. WIN and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St Louis, MO). Enzyme-linked immunosorbent assay (ELISA) kits for IL-1 $\beta$  and TNF- $\alpha$  from Pierce Biotechnology, Inc. (Rockford, USA). Western Blot Chemiluminescent Detection System (ECL) was from Amersham (Amersham Biosciences, Barcelona, Spain). Monoclonal anti-peroxisome proliferator-activated receptor antibody (PPAR- $\gamma$ ) (1:250) and polyclonal anti-cyclooxygenase-2 antibody (COX-2) (1:250) from Sigma Aldrich (Madrid, Spain). Monoclonal p65 antibody (p65) (1:250) from Santa Cruz Biotechnology (Madrid, Spain). Polyclonal anti-Cu/Zn superoxide dismutase antibody (Cu/Zn SOD) (1:250) from Assay Designs (Madrid, Spain). Monoclonal anti-tubuline (1:1000) from Cell Signaling (Beverly, MA, USA). All other reagents are analytical or culture grade purity.

## Primary culture of cortical astrocytes

All animals were handled according to the recommendations of the Bioethics Committee of the School of Medicine of the University of Valencia, Spain. Ethics committee specifically approved this study. Cortical astrocytes were isolated from rat fetuses of 21 days gestation. Fetuses were obtained by cesarean section and decapitated. Cerebral cortices were removed and triturated 10–15 times through a Pasteur pipette

with 10 ml DMEM. The cell suspension was filtered through nylon mesh with a pore size of 90  $\mu\text{m}$  and re-suspended in DMEM containing 20% fetal bovine serum (FBS), supplemented with L-glutamine (1%), HEPES (10 mM), fungizone (1%), and antibiotics (1%). Cells were plated on T75 culture flask and maintained in a humidified atmosphere of 5% CO<sub>2</sub>/95% air at 37°C during 15 days. After 4 days of culture, the FBS was maintained at 20% and after 1 week of culture, the FBS content was reduced to 10%, and the medium was changed twice a week. The purity of astrocytes was assessed by immunofluorescence using anti-glia fibrillary acidic protein (astrocyte marker; Sigma-Aldrich), anti-CD-68 (microglial marker; Serotec), anti-myelin basic protein (oligodendroglial marker; Sigma-Aldrich) and anti-microtubule-associated protein 2 (neuronal marker; Sigma-Aldrich). The astrocyte cultures were found to be at least 99% glial fibrillary acidic protein positive. No cells were found to express CD-68, myelin basic protein, or microtubule-associated protein-2.

## **Cell treatments**

Ten days after seeding, WIN (10  $\mu\text{M}$ ) was added to culture flasks. Twenty-four hours later, 10  $\mu\text{M}$  A $\beta$ <sub>1-42</sub> (toxic peptide) or A $\beta$ <sub>40-1</sub> (control peptide) (Sigma-Aldrich) were added to the flasks. A $\beta$ <sub>1-42</sub> concentration used in our study is in the range of toxic concentrations of the peptide [21,22]. Before incubation, the peptides were diluted in 100  $\mu\text{M}$  of phosphate-buffered saline (PBS) and incubated for 24 h at 37° C. Assays were performed 24 h after peptide addition.

## **MTT assay**

Cell viability was determined by MTT assay. The MTT assay is a well-established, widely used and easily reproducible method for the assessment of cell viability and cytotoxicity [23,24]. Astrocytes were plated in 96-well culture plate and incubated with



WIN during 24h. Subsequently, A $\beta$ <sub>40-1</sub> (control) and A $\beta$ <sub>1-42</sub> peptides were added to wells for another 24h. After cell treatments, the medium was removed and cells were incubated with red free medium and MTT solution [0.5 mg/ml, prepared in phosphate buffer saline (PBS) solution] for 4 h at 37°C. Finally, the medium was removed and formazan particles were dissolved in dimethyl sulfoxide (DMSO). Cell viability, defined as the relative amount of MTT reduction, was determined by spectrophotometry at 570 nm.

### **Cytokine determination, IL-1 and TNF $\alpha$**

Astrocytes were seeded as previously published [12]. At the time of assay, the red phenol medium was removed and replaced by PBS containing 1 mg/ml bovine serum albumin (BSA), either in the presence or absence of A $\beta$ <sub>1-42</sub> (10  $\mu$ M). IL-1 $\beta$  and TNF- $\alpha$  concentration (pg/ml) were ascertained using ELISA kits (Pierce Biotechnology, Inc.).

### **Western blot analysis**

Cultured cells were treated with lysis buffer and mechanically degraded to release the proteins. Protein concentration was determined using modified Lowry method [25]. Loading buffer (0.125 M Tris-HCl, pH 6.8, 2% SDS, 0.5% (v/v) 2-mercaptoethanol, 1% bromophenolblue and 19% glycerol) was added to protein sample and heated for 5 min at 95°C. Proteins were separated on SDS-PAGE gels and transferred to nitrocellulose membranes in a humid environment using a transfer buffer (25mM Tris, 190mM glycine, 20% methanol). Membranes were blocked with 5% milk in TBS (0.05% Tween-20) and incubated with primary antibodies overnight at 4°C. Membranes were washed 3 times with wash buffer TBS-T (TBS, 0.2% Tween-20) and incubated with a secondary anti-rabbit IgG or anti-mouse IgG (Cell Signalling Technologies Danvers, MA) antibody conjugated to the enzyme horseradish peroxidase (HRP) for 1 h. Membranes were washed three times and proteins were detected using the ECL method as specified by the

manufacturer. Autoradiography signals were assessed using digital image system ImageQuant LAS 4000 (GE Healthcare).

## **Statistical analyses**

Values are expressed as mean $\pm$ S.D. Differences between groups were assessed by one-way analysis of variance (ANOVA). Statistical significance was accepted at  $P \leq 0.05$ . Data sets in which  $F$  was significant were examined by a modified  $t$ -test.

## **Results**

### **Protective role of WIN on cell viability**

The role of WIN on cell viability was studied using MTT conversion assay. Figure 1A shows that incubation with WIN at different concentrations induced a significant increase in cell viability at 10  $\mu$ M. Consequently, that concentration was used in future experiments. Astrocytes previously incubated with 10  $\mu$ M  $A\beta_{1-42}$  for 24 h significantly decreased cell viability compared to control cells (Figure 1B). Furthermore, pretreating astrocytes during 48 h with WIN (10  $\mu$ M) prevented the decrease in cell viability induced by  $A\beta_{1-42}$  (WIN +  $A\beta$ ), conversely WIN (1, 2, 5 $\mu$ M) did not have any effect (Figure 1B).

### **WIN prevents IL-1 $\beta$ and TNF- $\alpha$ increase elicited by $A\beta_{1-42}$**

Cultured astrocytes were incubated with 10  $\mu$ M  $A\beta_{1-42}$  and proinflammatory mediators TNF- $\alpha$  and IL-1 $\beta$  were detected by ELISA.  $A\beta_{1-42}$  increased 4.5-fold IL-1 $\beta$  release (480.4 $\pm$ 150.3 pg/ml) compared with control (103.9 $\pm$ 82.9 pg/ml) (Figure 2A) and 2.4 fold TNF- $\alpha$  release (605.3 $\pm$ 103.4 pg/ml vs 210.5 $\pm$ 85.3 pg/ml in control group) (Figure 2B). Furthermore, WIN pre-treatment (10  $\mu$ M) prevented the increase in pro-inflammatory mediators induced by  $A\beta_{1-42}$  (Figure 2 A and B).

## **Effect of A $\beta$ <sub>1-42</sub> and WIN on p65 protein expression**

NF- $\kappa$ B, the pro-inflammatory transcription factor, is formed by different subunits. We measured p65 protein expression by western-blot. Incubation with A $\beta$ <sub>1-42</sub> increased p65 protein expression compared with control astrocytes (Figure 3), which was prevented by WIN pretreatment. ( $p < 0.05$  compared with A $\beta$ <sub>1-42</sub> treated astrocytes)

## **WIN prevents COX-2 and iNOS protein increase induced by A $\beta$ <sub>1-42</sub> peptide**

Incubation with A $\beta$ <sub>1-42</sub> significantly increased inflammatory proteins COX-2 (Figure 4A) and iNOS (Figure 4B) expressions compared to control. Furthermore, pretreating astrocytes with WIN prevented the effects produced by A $\beta$ <sub>1-42</sub>.

## **Effect of A $\beta$ <sub>1-42</sub> and WIN on PPAR- $\gamma$ protein expression**

Pro-inflammatory gene expression is downregulated by PPARs family [26]. We found that pretreatment with WIN (10  $\mu$ M) increased PPAR- $\gamma$  expression compared to control cells (Figure 5). Incubation with A $\beta$ <sub>1-42</sub> significantly decreased PPAR- $\gamma$  expression that was prevented by WIN pretreatment.

## **Effect of A $\beta$ <sub>1-42</sub> and WIN on Cu/Zn SOD and Mn SOD protein expression.**

Superoxide dismutase (SOD) is a key antioxidant enzyme. In our study, incubation with A $\beta$ <sub>1-42</sub> decreased Cu/Zn SOD expression in astrocytes in primary culture which was prevented by WIN pretreatment, evidencing that WIN could play a neuro-protective role against oxidative stress induced by A $\beta$ <sub>1-42</sub> peptide (Figure 6A). On the other hand, our results indicated that Mn SOD protein expression is increased in presence of A $\beta$ <sub>1-42</sub>.

Pretreatment with WIN did not prevent Mn SOD increase induced by A $\beta$ <sub>1-42</sub> (Figure 6B).

## Discussion

Oxidative stress and inflammation are the main mechanisms in the progression of various neurodegenerative diseases, including AD [27-30]. In our study, we determined different markers involved in inflammation and oxidative stress induced by the A $\beta$ <sub>1-42</sub> peptide in primary cultures of astrocytes, with the aim to assess the antioxidant and anti-inflammatory effects of cannabinoid WIN. We found that WIN significantly increased astrocytes viability compared to control cells. Furthermore, WIN prevented the decrease in astrocytes viability induced by A $\beta$ <sub>1-42</sub>.

It has been shown that cannabinoids preserve neurons from A $\beta$  exposure by activating MAP kinase cascade [31] and by anti-oxidative and anti-apoptotic effects [32]. Moreover, some studies demonstrated that cannabinoids protect glial cells from death [33,34]. Nevertheless, in cancer, where cells are highly proliferative and undifferentiated, treatment with cannabinoids can block cell proliferation in a dose dependent manner [35-38], demonstrating that the effects of cannabinoids on cell viability are probably dependent on cell type [39] and developmental stage [40].

Expression of CB1 [41] and CB2 [42] receptors in rat culture astrocytes have been published and also dual activation of both cannabinoid receptors by WIN 55,212-2 (the mixed non-selective CB1/CB2 agonist) in rat cortical astrocytes have been detected [41]. On the other hand, WIN confers its protective and anti-inflammatory effects against A $\beta$  injury through both CB1 and CB2 receptors [43]. Given that our results there is expression of both types of cannabinoid receptors (CB1 and CB2), it is likely that the effect of WIN observed in our study is due to the interaction with both types of receptors, consistent with published results by Fakhfouri and cols [44].

We found that WIN prevented the increase of inflammatory mediators IL-1 $\beta$ , TNF- $\alpha$ , NF- $\kappa$ B, iNOS and COX-2, as well as the decrease of the anti-inflammatory mediator

PPAR- $\gamma$  induced by A $\beta_{1-42}$  in astrocytes in primary culture. The inflammatory process is a characteristic mechanism in the development of AD, and pro-inflammatory agents are involved in the progression of cell damage [45,46,47,12]. Moreover, it is known that astrocytes participate in the inflammatory process induced by A $\beta_{1-42}$  [27,28,48]. Initially, inflammation is beneficial since it produces pro-inflammatory substances involved in tissue protection, limiting the survival and proliferation of cells exposed to toxic agents, such as A $\beta_{1-42}$  [49,50]. However, sustained inflammatory response could lead to neurotoxic damage or cell death [12,51,8]. NF- $\kappa$ B proteins are up-regulated in inflammation conditions such as astroglial activation induced by A $\beta_{1-42}$  oligomers [52]. In this regard, we found an increase in NF- $\kappa$ B/p-65 expression in astrocytes after addition of A $\beta_{1-42}$  that was prevented by WIN pretreatment. Valles and collaborators [53] found that the cytokine-receptor complex is able to bind to cytokines and other proteins of the extracellular matrix, producing inflammatory signals which could be important in pathologies such as Alzheimer's disease [53,54]. In agreement with our results, different authors have reported that cannabinoids mitigate neural cell activation in the neuroinflammatory response induced by A $\beta_{1-42}$ , reducing the levels of pro-inflammatory molecules such as IL-1 $\beta$ , TNF- $\alpha$ , COX-2 and iNOS [55,56,57]. Likewise, the activation of cannabinoid receptors diminishes the release of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in microglial cells [58,59,60] as well as COX-2 and iNOS [61]. Studies conducted in rats pretreated with the A $\beta$  peptide found that WIN prevented cognitive impairment, glial activation and neuronal loss [19,62,63], and also reduced COX-2, iNOS and TNF- $\alpha$  levels [63,64].

Kainu et al. [65] demonstrated for the first time the presence of mRNA and protein PPAR- $\gamma$  in CNS cells. Subsequent studies have detected PPAR- $\gamma$  expression in microglial and astrocytic cells [66,12]. PPAR- $\gamma$  agonists protect against A $\beta$ -induced

inflammatory and neuronal damage [67,68], thus making neurons and astrocytes potential therapeutic targets for PPAR- $\gamma$  ligands [69,12]. Astrocytes also express the largest levels of PPAR- $\gamma$  in the neural tissues [70,71]. As other authors [72,73,74], we found a decreased expression of PPAR- $\gamma$  in astrocytes treated with A $\beta_{1-42}$ . Esposito et al. [56] showed in neurons that cannabinoids may act as neuro-protective agents by PPAR- $\gamma$  activation. In this study, we demonstrate an increase in this protein expression in astrocytic cells previously incubated with WIN. Furthermore, we found for the first time that WIN prevents PPAR- $\gamma$  expression decrease induced by A $\beta_{1-42}$  peptide in astrocytes in primary culture. There is strong evidence to suggest that some cannabinoids can act on PPARs through either direct or indirect pathways. In order to directly act on nuclear transcriptional factors PPARs, exogenous cannabinoids need to pass through plasma membrane and be transported into nucleus which may involve certain membrane and intracellular transporters. However, we still cannot rule out that cannabinoids effects could be indirect through the binding of other cellular targets which in turn induces PPARs activation [75]. In fact, WIN attenuates amyloid-beta-induced neuroinflammation in rats through activation of cannabinoid receptors and PPAR- $\gamma$  pathway [44].

Different authors have demonstrated the role of oxidative stress in AD [76-79]. The cumulative damage caused by free radicals induces alterations in the activity or expression of antioxidant enzymes like catalase or SOD. These enzymes were found to be decreased in both CNS and peripheral tissues of AD patients [80,81]. In this sense, we demonstrate that Cu/Zn SOD is decreased in astrocytes treated with A $\beta_{1-42}$ . Our results are consistent with those reported by other authors, highlighting the role of oxidative stress in the development of AD [82]. New substances are under research to reduce damage caused by oxidative stress in this disease. Widely distributed in the body,

cannabinoids receptors were discovered few decades ago and are still under research [57]. Few studies address the effect of cannabinoids on oxidative stress. For instance, cannabinoids were found to prevent or antagonize oxidative stress toxicity in cortical neurons in cultures [83,84], and in lymphoblastic cells [85]. Studies with PC12 cells exposed to A $\beta$ <sub>1-42</sub> peptide demonstrated that cannabinoids reduced reactive oxygen species production and membrane lipid oxidation [86,32]. Our results provide evidence that A $\beta$ <sub>1-42</sub> decreases Cu/Zn SOD expression in astrocytes in primary culture, and pretreatment with WIN increases Cu/Zn SOD expression, preventing the decrease caused by A $\beta$ <sub>1-42</sub>. These findings indicate that cannabinoids could act as a protective agent against oxidative stress caused by A $\beta$ <sub>1-42</sub>. In Figure 7 the set of results are summarized. However our results indicate that A $\beta$ <sub>1-42</sub> elevated Mn SOD protein expression, increasing mitochondrial biogenesis mechanism, such as we previously published [74]. Pretreatment with WIN did not prevent Mn SOD overexpression induced by A $\beta$ <sub>1-42</sub>. Mn SOD plays a role in the adaptive response which protects brain cells from damage, as in the case of AD. In fact, Mn SOD preserves neurons against oxidative stress [87] and protects developing neurons from  $\beta$ -amyloid toxicity [88]. This enzyme catalyzes the conversion of superoxide radicals to molecular oxygen and H<sub>2</sub>O<sub>2</sub>, whereas glutathione peroxidase, peroxiredoxin reductase and catalase neutralize H<sub>2</sub>O<sub>2</sub>. Overexpression of Mn SOD induces cognitive recovery and reduces A $\beta$  levels in AD animal models [89]. Furthermore, Mn SOD deficiency increases  $\beta$ -amyloid levels and amyloid plaque burden, promoting the development of behavioural disturbances [90].

Preclinical data suggest a beneficial role of some cannabinoids for treatment of different diseases. Dronabidol, an oil-based solution of  $\Delta$ 9-THC, is used as anti-emetic and appetite stimulant [91].  $\Delta$ 9-THC also decreases agitation present in the advanced stage of AD [92]. In 2003, the FDA granted the patent for cannabinoids as antioxidants



and neuro-protectants (U.S. Department of Health and Human Services). Despite these promising preliminary results, the clinical utility of cannabinoids in AD is still to be determined [93].

## Conclusions

Taken together, our findings show that cannabinoid WIN increases cell viability and anti-inflammatory response in astrocytes in primary culture and prevents cell death induced by  $A\beta_{1-42}$ . Furthermore, WIN increases expression of anti-oxidant Cu/Zn SOD and is able to prevent inflammation induced by  $A\beta_{1-42}$  in astrocytes. In this sense, clinical studies are needed to evaluate the neuro-protective effects of cannabinoids in Alzheimer's disease.

## Conflict of Interest Statement:

The authors declare that there are no conflicts of interest.

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## Figure Legends

**Figure 1. Astrocytes viability.** (A) Astrocytes viability induced by WIN. Concentration-dependent viability of WIN (1, 2, 5, 10  $\mu$ M) was determined by MTT assay for 24 h. Data are means  $\pm$  SD for 4 independent experiments. \* $p$ <0.04 comparing WIN vs control cells. (B) Astrocytes viability in cells treated during 24 h with 10  $\mu$ M A $\beta$ <sub>40-1</sub> (control peptide, C), 10  $\mu$ M A $\beta$ <sub>1-42</sub> (A $\beta$ ) and WIN (1, 2, 5, 10  $\mu$ M) + 10  $\mu$ M A $\beta$ <sub>1-42</sub> (WIN + A $\beta$ ). Data are means  $\pm$  SD of 3 independent experiments. \* $p$ <0.05 vs control cells.

**Figure 2. IL-1 $\beta$  and TNF- $\alpha$  secretion.** WIN prevents the increase of IL-1 $\beta$  and TNF- $\alpha$  secretion caused by A $\beta$ <sub>1-42</sub> in astrocytes. Cells were incubated with 10  $\mu$ M A $\beta$ <sub>40-1</sub> (control peptide, C), 10  $\mu$ M A $\beta$ <sub>1-42</sub> (A $\beta$ ), 10  $\mu$ M WIN + 10  $\mu$ M control peptide (WIN) and 10  $\mu$ M WIN + 10  $\mu$ M A $\beta$ <sub>1-42</sub> (WIN + A $\beta$ ). Cell culture supernatants were harvested, and IL-1 $\beta$  (panel A) and TNF- $\alpha$  (panel B) secretion were determined by ELISA. Values are means  $\pm$  SD of replicate experiments from 4 independent astrocytes cultures. \* $p$ <0.05 vs control astrocytes. # $p$ <0.05 vs A $\beta$ <sub>1-42</sub> treated cells.

**Figure 3. p65 protein expression.** WIN 55, 212-2 prevents p65 expression induced by A $\beta$ <sub>1-42</sub> in astrocytes in primary culture. p65 and  $\alpha$ -tubulin expressions were determined by Western-blot in astrocytes treated for 24 h with 10  $\mu$ M A $\beta$ <sub>40-1</sub> (control peptide, C), 10  $\mu$ M A $\beta$ <sub>1-42</sub> (A $\beta$ ), 10  $\mu$ M WIN + 10  $\mu$ M control peptide (WIN) and 10  $\mu$ M WIN + 10  $\mu$ M A $\beta$ <sub>1-42</sub> (WIN + A $\beta$ ). A representative immunoblot of each protein is shown and tubulin was used as control amount of protein. Data are means  $\pm$  SD of 5 independent experiments. \* $p$ <0.05 vs control cells. # $p$ <0.05 vs A $\beta$ <sub>1-42</sub>.

**Figure 4. COX-2 and iNOS protein expression.** WIN prevents COX-2 and iNOS expression induced by A $\beta$ <sub>1-42</sub>. COX-2 (panel A), iNOS (panel B) and  $\alpha$ -tubulin expressions were determined by Western-blot in astrocytes treated for 24 h with 10  $\mu$ M A $\beta$ <sub>40-1</sub> (control peptide, C), 10  $\mu$ M A $\beta$ <sub>1-42</sub> (A $\beta$ ), 10  $\mu$ M WIN + 10  $\mu$ M control peptide (WIN) and 10  $\mu$ M WIN + 10  $\mu$ M A $\beta$ <sub>1-42</sub> (WIN + A $\beta$ ). A representative immunoblot of each protein is shown and tubulin was used as control amount of protein. Data are means  $\pm$  SD of 6 independent experiments. \* $p$ <0.05 vs control cells. # $p$ <0.05 vs A $\beta$ <sub>1-42</sub>.

**Figure 5. PPAR- $\gamma$  protein expression.** WIN induces PPAR- $\gamma$  expression in astrocytes in primary culture treated with A $\beta$ <sub>1-42</sub>. PPAR- $\gamma$  and  $\alpha$ -tubulin expressions were determined by Western-blot in astrocytes treated for 24 h with 10  $\mu$ M A $\beta$ <sub>40-1</sub> (control peptide, C), 10  $\mu$ M A $\beta$ <sub>1-42</sub> (A $\beta$ ), 10  $\mu$ M WIN + 10  $\mu$ M control peptide (WIN) and 10  $\mu$ M WIN + 10  $\mu$ M A $\beta$ <sub>1-42</sub> (WIN + A $\beta$ ). A representative immunoblot of each protein is shown and tubulin was used as control amount of protein. Data are means  $\pm$  SD of 4 independent experiments. \* $p$ <0.05 vs control cells. # $p$ <0.05 vs A $\beta$ <sub>1-42</sub>.

**Figure 6. Cu/Zn-SOD and Mn-SOD protein expressions.** WIN prevents Cu/Zn-SOD expression decrease in astrocytes in primary culture treated with A $\beta$ <sub>1-42</sub>. Cu/Zn-SOD, Mn-SOD and  $\alpha$ -tubulin expressions were determined by Western-blot in astrocytes treated for 24 h with 10  $\mu$ M A $\beta$ <sub>40-1</sub> (control peptide, C), 10  $\mu$ M A $\beta$ <sub>1-42</sub> (A $\beta$ ), 10  $\mu$ M WIN + 10  $\mu$ M control peptide (WIN) and 10  $\mu$ M WIN + 10  $\mu$ M A $\beta$ <sub>1-42</sub> (WIN + A $\beta$ ). A representative immunoblot of each protein is shown and tubulin was used as control amount of protein. Data are means  $\pm$  SD of 4 independent experiments. \* $p$ <0.05 vs control cells. # $p$ <0.05 vs A $\beta$ <sub>1-42</sub>.

**Figure 7. Preventive function of cannabinoid WIN on A $\beta$ <sub>1-42</sub>-induced toxic effects in astrocytes in primary culture.** Cannabinoid WIN 55,212-2 increases cell viability and anti-inflammatory response in cultured astrocytes and prevents inflammatory effects induced by A $\beta$ <sub>1-42</sub>.

Reviewer #1: I appreciate the efforts taken by the authors to adequately address the previous concerns with respect to this manuscript.

Following are the two minor issues that I have.

1. In the figure legend for Figure 7. modify the last line to match with the conclusion from this study (delete the part that WIN 55,212-2 prevents oxidative stress, since no direct measure of oxidative stress was done).

Modifications in Figure 7 have been done to match with the conclusion.

2. In revised manuscript, authors show that 10  $\mu$ M A $\beta$ 1-42 increases MnSOD protein expression. In the discussion section (page 14), can the authors further speculate regarding the conclusion of this finding with respect to mitochondria and oxidative stress during Alzheimer's disease, since mitochondria are the major organelles producing superoxide radicals leading to oxidative stress. To adequately answer this, apart from astrocytes, which are the focus of this study, what is known regarding mitochondria and oxidative stress in Alzheimer's disease in other cells like neuronal cells? And does more MnSOD mean more H<sub>2</sub>O<sub>2</sub> production, that might be a mediator of the oxidative stress ?

A paragraph about all suggestions indicated by the referee has been added in discussion.