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Synaptic Silencing and Plasma Membrane Dyshomeostasis Induced by Amyloid-β Peptide are Prevented by *Aristotelia chilensis* Enriched Extract

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Abstract. Alzheimer's disease (AD) is characterized by the presence of different types of extracellular and neurotoxic aggregates of amyloid-β (Aβ). Recently, bioactive compounds extracted from natural sources showing neuroprotective properties have become of interest in brain neurodegeneration. We have purified, characterized, and evaluated the protective potential of one extract enriched in polyphenols obtained from *Aristotelia chilensis* (MQ), a Chilean berry fruit, in neuronal models of AD induced by soluble oligomers of $A\beta_{1\text{-}40}$. For example, using primary hippocampal cultures from rats (E18), we observed neuroprotection when the neurons were co-incubated with Aβ (0.5 μM) plus MQ for 24 h (Aβ = 23 ± 2%; Aβ + MQ = 3 ± 1%; $n = 3$). In parallel, co-incubation of Aβ with MQ recovered the frequency of Ca^{2+} transient oscillations when compared to neurons treated with Aβ alone (Aβ = 72 ± 3%; Aβ + MQ = 86 ± 2%; $n = 5$), correlating with the changes observed in spontaneous synaptic activity. Additionally, MAP-2 immunostaining showed a preservation of the dendritic tree, suggesting that the toxic effect of Aβ is prevented in the presence of MQ. A new complex mechanism is proposed by which MQ induces neuroprotective effects including antioxidant properties, modulation of cell survival pathways, and/or direct interaction with the Aβ aggregates. Our results suggest that MQ induces changes in the aggregation kinetics of Aβ producing variations in the nucleation phase (Aβ: $k_1 = 2.7 \pm 0.4 \times 10^{-3}$ s^{-1} MQ: $k_1 = 8.3 \pm 0.6 \times 10^{-3}$ s^{-1}) and altering Thioflavin T insertion in β-sheets. In conclusion, MQ induces a potent neuroprotection by direct interaction with the Aβ aggregates, generating far less toxic species and in this way protecting the neuronal network.

Keywords: Alzheimer's disease, amyloid-β peptide, antioxidant, hippocampal neurons, maqui, nutraceuticals, polyphenols

INTRODUCTION

Alzheimer's disease (AD) is a complex and progressive clinical condition where the main consequences are related to cognitive and memory dysfunctions [1, 2] due to irreversible neurodegeneration, synaptic dysfunction, and neuronal death. This pathology is one

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of the four most common causes of death [3]. It is currently believed that AD is a multifaceted disease characterized by an abnormal increase in amyloid-β peptide (Aβ) levels. These increased levels have been related to different hypotheses trying to explain the physiopathology of the disease, for example: AβPP mutation, mutations in the presenilin gene 1 and gene 2, and others [4–6]. All these factors can increase Aβ levels [7] and appear to be important in the onset of toxic effects and could be considered a target for the development of new drugs or treatment strategies to slow or stop the progression of the disease. Therefore, it is relevant to come to a better understanding of the cellular and molecular mechanisms that trigger the development of AD.

Aβ peptides are generated by the action of β- and γ-secretases [8] obtaining "long" ($A\beta_{1\text{-}42/43}$) and "short" peptides ($A\beta_{1\text{-}40}$), respectively [5, 9–11]. Progressively, the main attention has been taken away from senile plaques and focused on soluble oligomers (SO) formed by $A\beta_{1\text{-}40/42}$ [12–14]. Despite that trophic effects have been demonstrated in the presence of Aβ monomers [15], it is currently believed that the "soluble oligomeric" aggregated form of Aβ (SO-Aβ, 56 kD) is the initial agent responsible for the dyshomeostatic alterations that induce neurodegenerative effects [16–20], and these neurotoxic effects are found in the low micromolar range [19, 21–23]. Recently, the ability of the peptide to form non-selective pores in the neuronal membrane was shown [23, 24], supporting data previously published in models *in vitro* [25] that help to reasonably explain the overload of cytosolic Ca^{2+} and release of essential molecules such as ATP [26].

AD continues to be a biomedical challenge due to the absence of effective therapies that could deter the progression of the disease, and also due to the lack of biomarkers that would allow for an early detection [27]. Therefore, any clinical or pharmacological strategy that could help to slow down disease advancement or be considered as a parallel treatment would be a significant alternative for these patients.

Available studies suggest that foods, such as nutraceuticals or those enriched with polyphenols, display bioavailability in several types of body tissues [28]. In addition, they improved cognition in several animal models [29] suggesting that they could be beneficial in the prevention of AD. NSAIDs, estrogen, HMG-CoA reductase inhibitors (statins), tocopherol (vitamin E), and antibodies [30] have been tested as potential anti-AD drugs, but side effects, adverse reactions, and poor efficacy have delayed drug development. In parallel, an important number of studies have shown that nutrient compounds present in fruits, plants, and other natural foods have beneficial properties as potential neuroactive tools [3, 26, 31–36]. More detailed studies have demonstrated the effects of polyphenols in various neurodegeneration models [26, 29, 35, 37]. For example, resveratrol has been shown to have some anti-amyloidogenic effects on Aβ aggregation [38–40] and also modulates signaling pathways such as MAP kinases and pCREB [41, 42]. All this evidence confirms the usefulness of compounds obtained from natural sources and their potential beneficial properties against Aβ toxicity.

In this work, we studied the effects of polyphenol-enriched extracts from a native Chilean shrub called Maqui (*Aristotelia chilensis*) in rat hippocampal neurons exposed to SO-Aβ to evaluate neuroprotective properties on SO-Aβ toxicity. This typical berry (MQ) from the south of Chile has several properties including anti-inflammatory actions, α-glucosidase/α-amylase inhibitor activity [43], and a potent antioxidant capacity based on its high concentration of polyphenolic compounds [44]. For these reasons, we decided to examine the properties of one extract of *Aristotelia chilensis* enriched in polyphenols on hippocampal neurons as a model for AD.

MATERIALS AND METHODS

Hippocampal cultures

18-19 days pregnant Sprague-Dawley rats were treated in accordance with regulations established by NIH and the Ethics Committee at the University of Concepción. Primary cultures of embryonic hippocampi were plated at 250,000 cells/ml on coverslips coated with poly-L-lysine (Sigma Aldrich, St. Louis, MO, USA). Cultures were maintained at 37°C with 5% CO_2. Culture medium was replaced every 3 days and consisted of 90% minimal essential medium (MEM; Gibco, Grand Island, NY, USA), 5% heat-inactivated horse serum, 5% fetal bovine serum and N3 (mg/ml: BSA 1, Putrescine 3.2, Insulin 1, Apotransferrin 5, Corticosterone 0.5; (μg/ml: Sodium selenite 5, TH3 0.5, Progesterone 0.6)). Experiments were performed at 10–12 DIV in control and treated neurons.

PC12 cells

PC12 cells from ATCC (Manassas, VA, USA) were utilized in experiments with a 96-well plate

reader. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. The cells were incubated under standard conditions (37°C, 5% CO_2) and when 80% confluence was achieved, the cells were treated with 0.25% trypsin for 10 min, washed, and resuspended in HyQ DMEM/High-Glucose (Hyclone, Logan, UT, USA) with 5% fetal bovine serum (Hyclone), 2 mM L-glutamine (Gibco), and 1% penicillin-streptomycin (Gibco). The cells were then plated at a concentration of 50,000 cells/well for experiments using the plate reader (96 wells, NOVOstar Labtech) and used 24 h after plating under experimental conditions similar for neurons.

Aristotelia chilensis extract

Enriched-MQ extracts were obtained from the fresh berries produced by the *Aristotelia chilensis* plant locally harvested, using a mixture of solvents (ethanol/water) and denominated MQ. To obtain the enriched polyphenol extracts (MQ), we used an XAD-7 Amberlite® adsorber resin (Merck, Germany) and an XAD-2 Amberlite® adsorber resin (Supelpack™-2, Sigma-Aldrich). This method resulted in an extract having 0.39% of polyphenols for each 100 g of fresh fruit, where the main components were myrcetin (0.060%) and ferulic acid (0.148%). Once this MQ extract was shown to have activity, it was characterized to determine its polyphenol composition through HPLC techniques. The final MQ extract was dried and diluted in DMSO to a final concentration of 810 mg/L. Different dilutions from 1 : 10 to 1 : 100000 were prepared daily in external solution containing (in mM): 5 $CaCl_2$, 100 NaCl, 45 TEA-Cl, 10 HEPES, 5.5 KCl, 10 glucose.

Immunofluorescence

The subcellular distribution of different targets in the neurons were detected using primary antibodies to SV2 and MAP2 and stained with specific secondary antibodies (Santa Cruz Biotechnology, USA); while the spatial localization of Aβ was determined using a fluorescent Aβ form ($Aβ_{1-40}$ TAMRA-labeled, Anaspec). Images were acquired with a confocal microscope (Nikon, Japan), and processed and quantified with Image J Software.

Cell viability assay

Cell cultures were incubated with MTT solution (1 mg/ml) for 30 min, and precipitated MTT was dissolved using isopropanol cooled for 15 min. Absorbance was measured in a multiplate reader (NovoStar, LabTech BMG, Germany) at two wavelengths: 560 nm and 620 nm, and the ratio was quantified using NovoStar Software for the different experimental conditions [45–47]. Similar results were obtained with other techniques, such as Alamar blue and LDH release assays (data not shown), however, we chose MTT because it is associated to mitochondrial dysfunction.

Spontaneous Ca^{2+} transients

Spontaneous Ca^{2+} transients were measured with 3 μM Fluo4-AM® (Invitrogen, USA). Hippocampal neurons were placed on coverslips coated with poly-L-lysine (Sigma-Aldrich) and incubated with the dye for 30 min at 37°C. The neurons were then washed twice and mounted on a perfusion chamber on a microscope (Nikon, TE 2000, Japan). The Fluo4-AM® was excited with a band pass filter at 480 nm wavelength and the emission recovered at 535 nm. Changes in cytosolic Ca^{2+} were registered with an EM-CCD camera (iXon ANDOR, USA) and a Lambda 10-B (Sutter Instruments, USA) interface. Each image was recorded every 0.5–1 s with a time exposure of 100 ms. Image analysis was made with an Imaging Workbench 6.0 software (Indec System, USA). Differential Ca^{2+} increase in the cell was measured in the cytosolic regions.

Electrophysiology

Spontaneous postsynaptic currents were recorded in hippocampal neurons during 2–4 min using patch clamp techniques (Axon amplifier 200B, Molecular Devices) in the voltage clamp mode (–60 mV holding potential) and whole cell configuration. These recordings were made in neurons preincubated during 24 h in the presence or absence of MQ extracts (0.81 mg/ml) with Aβ (0.5 μM). Different parameters such as frequency, amplitude, rise time (10–90%) and decay time constant (between 90–10% of decay) were determined and analyzed with MiniAnalysis 5.0 software (Synaptosoft, Inc, USA). From the resulting data, cumulative or frequency histograms were generated. For electrophysiological recordings, the pipette solution contained (in mM): 140 KCl, 10 BAPTA,

10 HEPES (pH 7.4), 4 $MgCl_2$, 0.3 GTP and 2 ATP-Na_2, 300 mOSM.

Electron microscopy

Aβ aggregates (80 μM) were prepared in the presence or absence of MQ extract (0.81 mg/ml) for 2 h at 37°C with 200 rpm agitation, and then placed on a 200 mesh nickel grid pre-treated with Formvar and shaded with charcoal. The presence of Aβ was confirmed by immunogold. The primary antibody recognized Aβ (Santa Cruz Biotechnology, USA) and the secondary antibody was an anti-mouse IgG conjugated to a 5–10 nm gold nanoparticle and phosphotungstic acid was used for contrast. The images were taken with a JEM 1200 EXII electron microscope (Jeol, Japan) operated at 80 kV.

Thioflavin T binding assay

Aβ aggregation was performed in a 96-well plate in the presence or absence of increasing concentrations of MQ (0.081, 0.27, 0.81, 2.7, 8.1 mg/ml) in PBS buffer with 20 μM of Thioflavin T (ThT, Sigma-Aldrich). The aggregation process was followed by fluorescence measurements of the Thioflavin T-Aβ complex (ex: 440 nm, em: 485 nm) every 3 min for 4 h. The plate was kept at 37°C with an orbital agitation of 200 rpm.

Data analysis

Statistical analyses were performed using Student's *t*-test or ANOVA test and are expressed as arithmetic mean ± SE. Values of $p < 0.05$ were considered statistically significant. All data analyses were made using GraphPad Prism 5.0 software.

RESULTS

Changes in the viability and morphology of the neuronal network induced by $A\beta_{1\text{-}40}$ were prevented by MQ

In order to examine the effect of chronic incubation with Aβ peptide on the neuronal function, rat hippocampal cells (10 DIV) were treated for 24 h with SO-Aβ (0.5 μM). After this time, neuronal cell viability was quantified using the MTT technique (Fig. 1A). The results showed that SO-Aβ decreased cell survival by 23 ± 2% at 24 h, whereas co-incubation with MQ partially prevented the toxicity of Aβ resulting in a cell death of only 2 ± 1.3%. In addition, we used an antibody against MAP-2 to evaluate neurite extension. Figure 1B shows the effect of Aβ on neuronal network as reflected by a reduced number in neuronal branching. Co-incubation with MQ (0.81 mg/ml) prevented the loss of branching produced by the peptide. This effect was better observed in the amplified images (button stack) where it is possible to see the recuperation of the branching in the presence of MQ with greater detail. Furthermore, in the presence of Aβ not only was there a loss of dendritic processes, but there was a thickening in the primary processes possibly due to hyperphosphorylation of the tau protein, with an increase in the thickness about two and a half fold as compared to control (Fig. 1C).

MQ prevents deterioration in the activity of the neuronal network induced by SO-Aβ

The decrease in cell viability observed with MTT was associated to a reduction in synaptic activity and spontaneous intracellular activity of Ca^{2+} because both depend on neuronal connectivity [48]. Electrophysiological experiments revealed that spontaneous synaptic currents were significantly decreased in the presence of SO-Aβ. For example, Fig. 2 demonstrates electrophysiological recordings done with patch clamp (voltage clamp/whole cell) from control neurons and those incubated with SO-Aβ. The results show that the synaptic activity was decreased to 47 ± 8% with respect to control in the presence of 0.5 μM SO-Aβ, whereas co-incubation with MQ was able to recover the frequency of synaptic currents to 138 ± 23%, indicating that MQ is not only capable of conserving the neurite structure, but can also preserve its functionality (Fig. 2B). In addition, Fig. 3A demonstrates recordings of intracellular calcium transients in control condition and treatment with SO-Aβ. The data show that chronic incubation with SO-Aβ greatly decreased the amplitude and frequency of the calcium transients by 29 ± 3% with respect to control, whereas co-incubation with MQ partially inhibited the effect of SO-Aβ and only displayed a decrease in frequency by 14 ± 2% with respect to control (Fig. 3B). These results suggest that the neuronal network activity was protected by the MQ extract against the toxicity of SO-Aβ in agreement with Fig. 1.

Effects of SO-Aβ and the MQ extract on the exocytosis machinery

As suggested above, the protective effect of MQ could be related to modulation of one of the

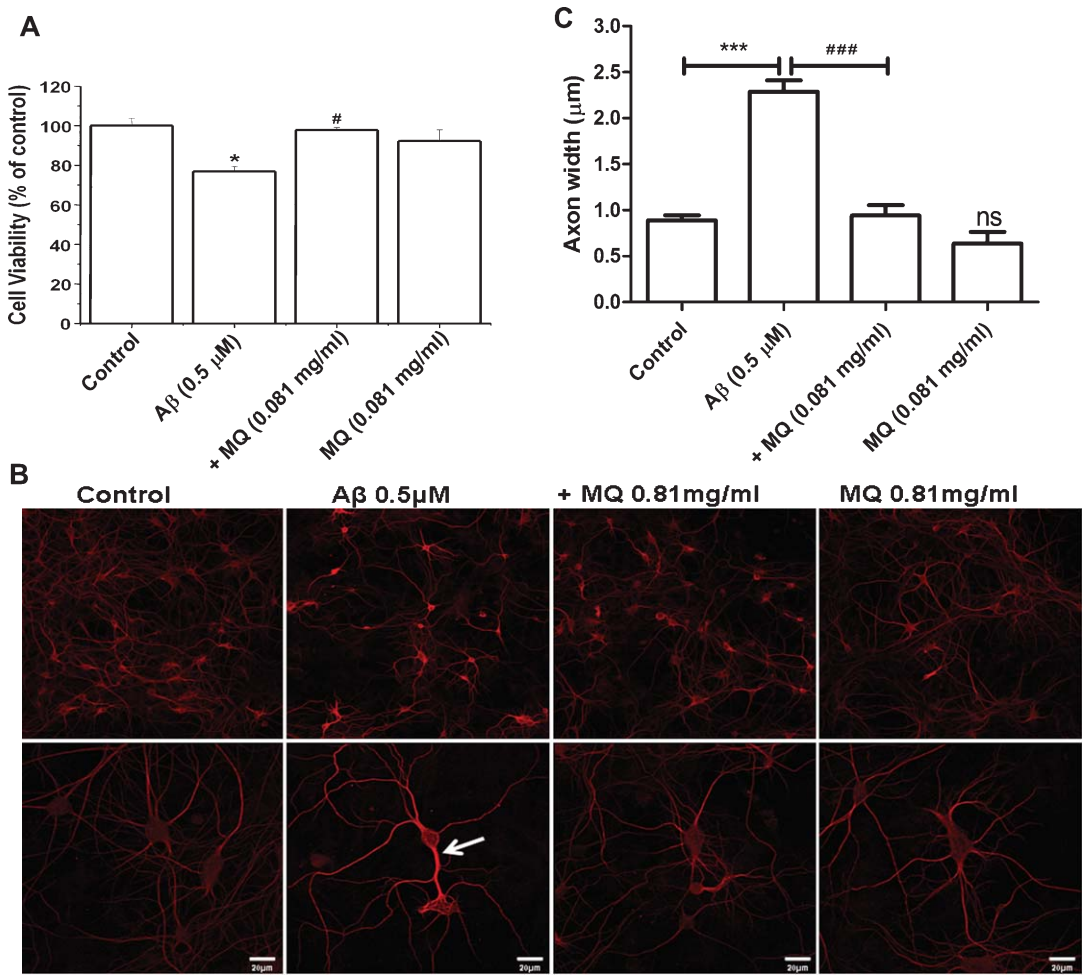

Fig. 1. Cell death and morphological changes induced by Aβ are prevented by the MQ extract. A) Soluble oligomers of Aβ (0.5 μM, 24 h) induced neuronal death; co-incubation with MQ (0.81 mg/ml) extract showed a prevention in Aβ toxicity ($n = 3$). B) Changes in neuronal network morphology induced by Aβ (0.5 μM, 24 h) were observed. Reduction in dendritic processes and thickening in axons (white arrow) were partially prevented by MQ (0.81 mg/ml) with recovery of the dendritic network. The images were obtained from primary hippocampal cultures and stained with MAP2 (scale bar 20 μm, $n = 3$). C) Quantification of neuronal axon width shown for images in B ($n = 3$, at least 4 neurons by field and condition for each n). $^{*}p < 0.05$ respect to control; $^{***}p < 0.001$ respect to control; $^{\#}p < 0.05$ respect to Aβ; NS – non significant.

ionic currents (excitatory or inhibitory) responsible for synaptic transmission, or to an effect in the expression level of proteins responsible for maintaining this synaptic transmission and neurotransmitter release. Therefore, we decided to study the effect of SO-Aβ and MQ on key proteins involved in exocytosis, such as SV2. Figure 4A shows confocal images of hippocampal neurons marked with an antibody that recognizes MAP2, a neuronal protein (red), and SV2, a synaptic vesicle protein (green). The data revealed that SO-Aβ (0.5 μM, 24 h) significantly decreased the immunoreactivity of SV2 in the neurons, suggesting an interference with the release mechanism for neurotransmitters, which correlates with the reduction in the frequency of synaptic currents that were observed in our electrophysiological experiments. However, when the neurons were co-incubated with MQ (0.81 mg/ml), the immunoreactivity for SV2 was recovered (Fig. 4A). Quantification of SV2 immunoreactive puncta for the different experimental conditions demonstrated that SV2 immunoreactivity in the presence of Aβ alone was $51 \pm 6\%$, but increased to $88 \pm 6\%$ when the MQ extract was present (Fig. 4B). These results support the idea that the MQ extract plays a role in the preservation of synaptic transmission.

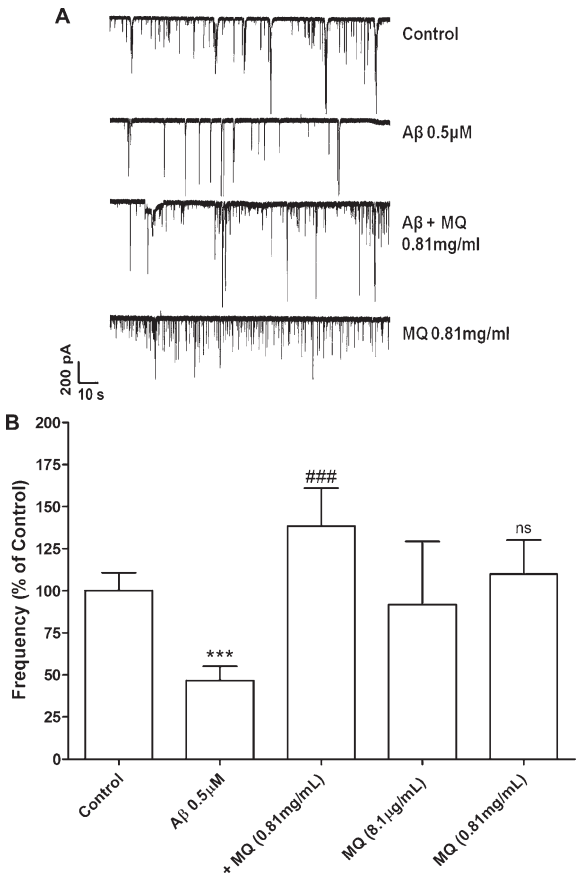

Fig. 2. The reduction in electrophysiological synaptic activity induced by Aβ was prevented by MQ. A) Representative traces of Ca^{2+} transients in control cells (upper), cells treated with Aβ (0.5 μM, middle upper), cells treated with Aβ plus MQ (0.81 mg/ml, middle lower), and cells treated with MQ (0.81 mg/ml, lower). B) Aβ disrupted the neuronal network reducing the synaptic communication and thus reducing the frequency of events, while MQ was able to partially recover the frequency restoring the neuronal network connection ($n = 3$). $^{*}p$ respect to control; $^{\#}p$ respect to Aβ; $^{***}p < 0.001$ respect to control; $^{\#\#\#}p < 0.001$ respect to Aβ.

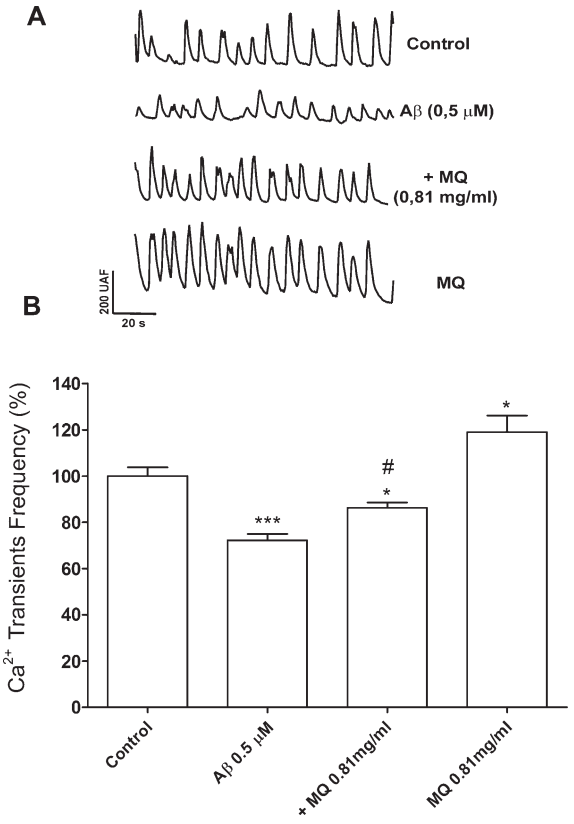

Fig. 3. Aβ changed the frequency of Ca^{2+} transients that was partially prevented by MQ. A) Representative traces of synaptic activity in untreated neurons (upper), neurons treated with Aβ (0.5 μM, middle upper), neurons treated with Aβ plus MQ (0.81 mg/ml, middle lower), and neurons treated with MQ (0.81 mg/ml, lower). Scale is 200 <AUF and 10 s ($n = 16$). B) Quantification of synaptic activity frequency revealed a decrease in frequency in the presence of Aβ (46.7 ± 8.44%), while MQ displayed a blocking effect and enhanced the communication in neurons treated with Aβ 0.5 μM (138.4 ± 22.5%). $^{***}p$ respect to control, $^{\#\#\#}p$ respect to Aβ, ns: not significant; $^{*}p < 0.05$ respect to control; $^{\#}p < 0.05$ respect to Aβ.

Interaction of SO-Aβ with the plasma membrane and blockade by the MQ extract

Recent studies from our laboratory and others [23, 25, 49] have demonstrated the interaction between the Aβ peptide and the plasma membrane through formation of a membrane pore that is permeable to Ca^{2+} that would be responsible for the early homeostatic dysregulation of the cell leading to apoptosis and neuronal death. Additionally, we have shown that essential molecules such as ATP are able to leak through this non-selective pore [26]. Therefore, we decided to evaluate the possibility that MQ could be able to intervene early on in the toxicity mechanism of SO-Aβ.

Figure 5A shows neurons marked with MAP2 (red) and with fluorescent SO-Aβ (green), and it can be seen that the fluorescence associated to Aβ near the neuronal membrane (bottom merge) is significantly decreased when MQ is co-incubated with the peptide with respect to the fluorescent signal of the peptide alone (upper merge). This was confirmed by the inability of the peptide to increment capacitative transients in the presence of MQ during electrophysiological recordings (cell attached/patch perforated). The increase in these transients is an indication of the electrical connection between the intracellular matrix and recording electrode which increases when the cell changes from a cell attached configuration to a patch perforated configuration (Fig. 5B). This did not occur with co-incubation of

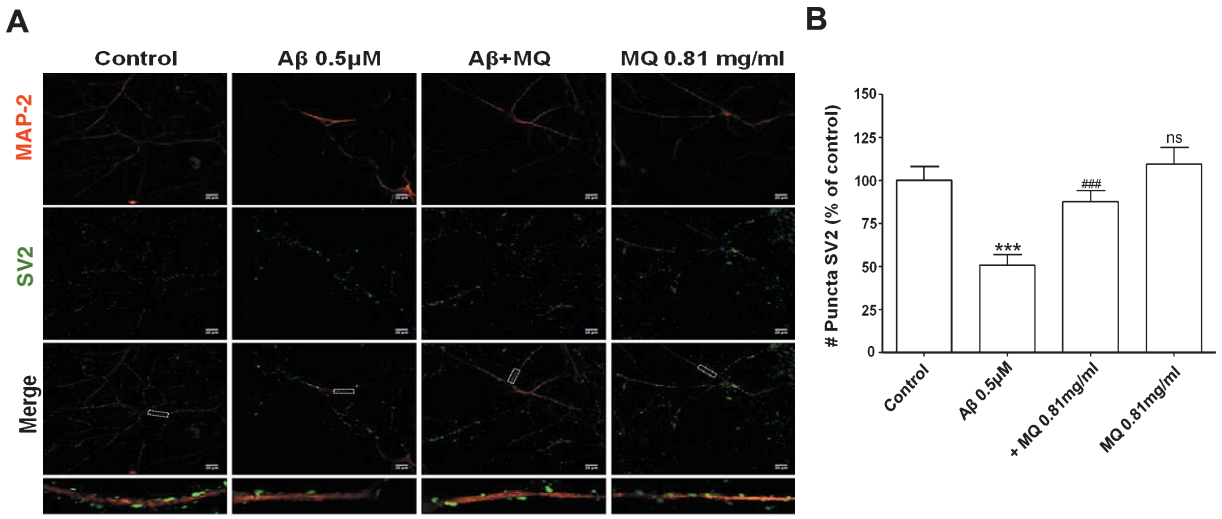

Fig. 4. MQ prevented the reduction in presynaptic proteins induced by Aβ. A) Confocal images (60X) obtained from primary hippocampal cultures and stained for MAP2 (red) and SV2 (green). Neurons were treated with Aβ (0.5 μM) and MQ (0.81 mg/ml). The puncta for SV2 were analyzed in ROI of 30 μm (white rectangle). Scale bar: 20 μM. B) Quantification of SV2 puncta in the primary processes. Aβ induced a decrease in the number of puncta for the presynaptic protein SV2 (50.7 ± 6.2%), while co-incubation with MQ prevented this effect (87.7 ± 6.3%), ($n = 3$), ***respect to control, ###respect to Aβ, ns: not significant.

the MQ extract which displayed values similar to those of control [49]. These results confirm the idea that MQ could be interfering with the perforating properties of Aβ.

Effects of the MQ extract on the type of SO-Aβ aggregates

Since the cytotoxicity of the Aβ peptide was altered in the presence of the MQ extract (Figs. 1–3), and in addition, there was a decrease in the binding capacity of the peptide to the plasma membrane (Fig. 5A), perhaps the MQ extract was altering the structural features of the aggregates. In order to study this possibility, electron micrograph images of the Aβ peptide marked with a specific Aβ antibody and conjugated to gold nanoparticles were analyzed. As shown in Fig. 6A (upper panels), the peptide formed small aggregates of diverse structures and sizes that are attributed to the toxicity reported in our study. However, when the MQ extract was incorporated into the aggregation process, it was possible to observe a change in the structural makeup of the aggregates with respect to those from control (lower panels). Moreover, it was possible to find peptide aggregates having different structures and sizes in the absence or presence of MQ at the lowest magnification analyzed (lower left panel) as well as at the highest magnification (right). These results were confirmed by kinetic studies of Thioflavin T insertion in the β-sheets of the amyloid peptide (Fig. 6B). The data show that incorporation of Thioflavin T to the peptide was decreased in the presence of the MQ extract in a concentration-dependent manner inducing changes in the aggregation kinetics of Aβ and producing variations in the nucleation phase (Aβ: $k_1 = 2.7 \pm 0.4 \times 10^{-3}$ s^{-1}; MQ: $k_1 = 8.3 \pm 0.6 \times 10^{-3}$ s^{-1}) and altering Thioflavin T insertion in β-sheets. In addition, fluorescent studies using Thioflavin T with Aβ alone and in the presence of MQ demonstrated that the extract significantly decreased Thioflavin T fluorescence (Fig. 6C). Altogether, these data support the idea that the MQ extract was capable of altering the identity of the Aβ peptide aggregates.

DISCUSSION

Using a cellular model based on cultured hippocampal neurons, we found a significant degree of neuroprotection when neurons were co-incubated with Aβ plus an MQ extract enriched in polyphenols for 24 h. This was detected as a recovery of spontaneous synaptic activity and preservation of the neuritic tree (by MAP-2 immunostaining), suggesting that the toxic effect of Aβ was prevented in the presence of MQ.

It was previously shown that Aβ inserts in the plasma membrane and induces a strong homeostatic cellular dysregulation [23, 24] leading to a cytosolic Ca^{2+} overload accompanied by an early enhancement of neurotransmitter release and a delayed state of

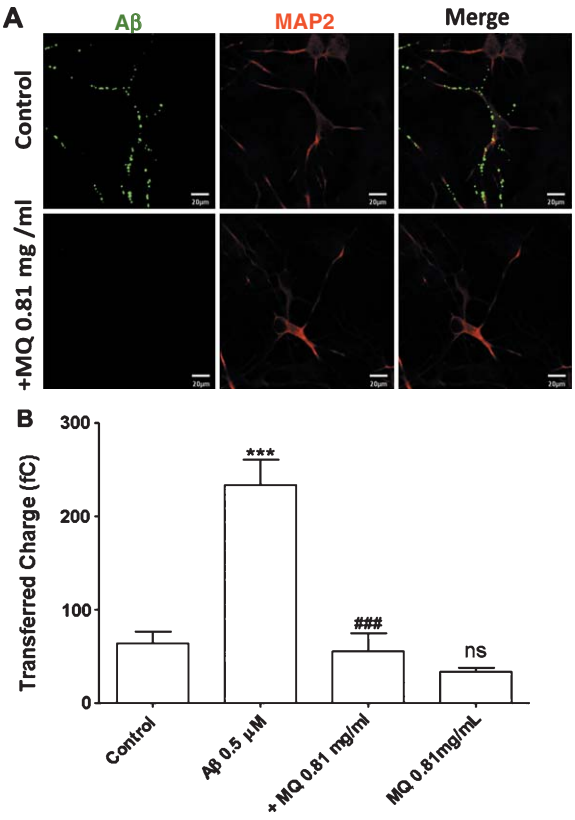

Fig. 5. The Aβ-plasma membrane interaction is partially blocked by MQ. A) Confocal images acquired on an equatorial plane at 60X to evaluate the Aβ-plasma membrane interaction show that the MQ extract diminished the binding of fluorescent Aβ (0.5 μM, green) to membrane neurons (marked with MAP2, red). B) Changes in capacitative transients (electrophysiological recordings) induced by Aβ and quantified by transferred charge were prevented by the MQ extract ($n = 4$). These capacitative modifications are directly correlated to the changes from cell attached to patch perforated configuration. ***respect to control, $^{###}$respect to Aβ, ns: not significant.

synaptic vesicle depletion, or synaptic failure. These changes are temporally associated to increases in post-synaptic Ca^{2+} transients (min-h) that are followed by delayed neuronal silencing reflecting a state of functional impairment. In this model, neuronal death follows prolonged membrane and ionic dysfunctions ($\geq$24 h with Aβ). These effects of Aβ were not associated to changes in ionic current parameters nor to generation of macroscopic membrane responses and only detected as changes in capacitative transients supporting the idea of Aβ insertion in the membrane [24].

In the present study, we showed that an extract from *Aristotelia chilensis* enriched in polyphenols had a direct effect on the Aβ-mediated toxicity at the cellular level. The main finding of our study was the capability of the polyphenols present in this native berry from the South of Chile to promote neuroprotection by mechanisms distinct to those classically associated to polyphenols. For example, it is believed that polyphenol actions are based on their ability to neutralize free radicals generated in the later stages of the toxic process of the peptide. On the other hand, our data showed that: 1) The MQ extract prevented synaptic failure and cell death induced by Aβ in primary hippocampal cultures. It also maintained neurite arborization which was significantly reduced by the peptide (Figs. 1 and 2); 2) The MQ extract improved neuronal network transmission as reflected by cytosolic Ca^{2+} transients, electrical synaptic activity and secretory machinery proteins (Figs. 2–4); and 3) The MQ extract altered the normal aggregation of Aβ, producing larger aggregates, and also produced changes in the normal structure of the complexes as shown by inhibition of Thioflavin T binding (Figs. 5 and 6). Previous studies from our and other laboratories reported that Aβ is able to form membrane pores that can alter the ionic homeostasis, especially that of intracellular calcium [23, 49]. The present results suggest that the MQ extract interferes with this pore formation and prevents the changes on functional neuronal parameters preceding neurotoxicity and cell death.

All these data indicate that the MQ extract can interfere with the formation of toxic Aβ species inducing a loss in its perforating properties (see Fig. 7). This in turn prevents the ionic dysregulation and the cascade of toxic effects, including reduction in SV2. These findings provide a mechanism to understand how natural compounds can prevent AD, suggesting a potent action in the initial stages of the peptide aggression on the neurons. This is contrary to the classic mechanism described for the antioxidant capacity of these polyphenolic molecules to neutralize free radicals; a mechanism resulting in a lower level of neuroprotection to prevent cell death since the phenomenon of oxidative stress occurs much later in the process of neuronal death. However, it cannot be ruled out that during prolonged periods of time the formation or presence of aggregate species having large sizes could be a continual source for smaller oligomers that finally deplete the capacity of MQ to efficiently block the toxicity of the peptide at chronic time periods.

Experimental evidence reinforce studies proposing the benefits of a diet rich in fruits and natural compounds [37, 39, 50–56]. Additionally, this study showed that polyphenols present in the *Aristotelia chilensis* extract were able to deter cell dysfunction, suggesting that a food supplement used as a nutraceutical preparation could inhibit Aβ aggregate formation

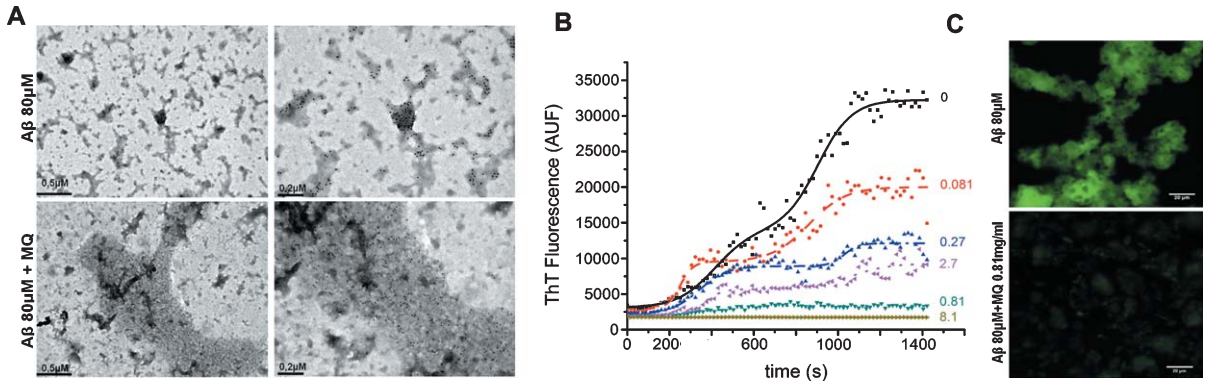

Fig. 6. MQ extract alters the aggregation process of Aβ. A) The left upper panel shows the normal ultrastructure of Aβ stained with gold nanoparticles, showing clusters of peptide roughly of the same size. The black dots are gold nanoparticles conjugated to an antibody specific for Aβ. The left bottom image shows a representative aggregate obtained in the presence of MQ. These structures are larger and more compact than control Aβ. The image on the right shows an enlargement of the central field for each condition. Scale bars: 500 nm and 200 nm, respectively ($n = 3$). B) Insertion kinetics for Thioflavin T on Aβ aggregates were inhibited by the MQ extract. Time course of Aβ aggregation followed by Thioflavin T insertion alone (black line), or co-aggregated with different MQ concentrations (in mg/ml): 0.081 (red), 0.27 (blue), 2.7 (fuchsia), 0.81 (green), and 8.1 (brown). C) The upper panel shows a representative fluorescent image of Aβ (80 μM) aggregates stained with Thioflavin T; the lower panel is an example of fluorescent images of Aβ aggregates in the presence of MQ (0.81 mg/ml); Scale bar: 20 μm ($n = 3$).

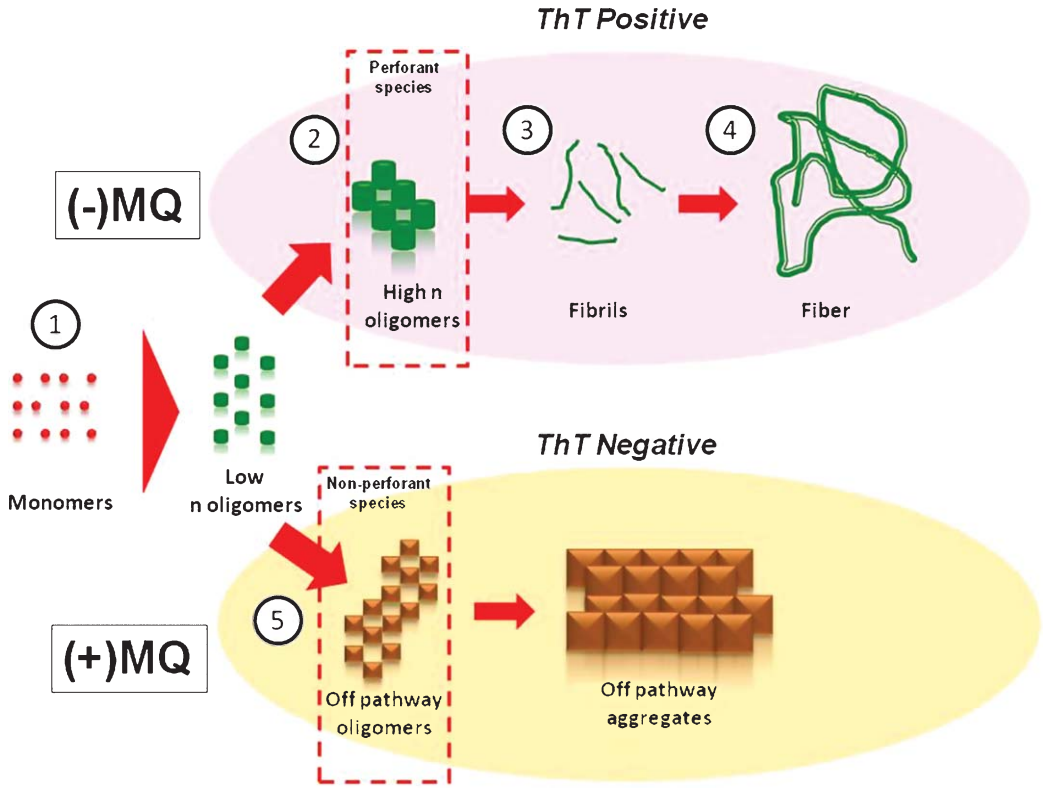

Fig. 7. Scheme representing the proposed effects of MQ on Aβ aggregates. Aβ aggregation is a nucleation dependent process (1). The formation of low n oligomers induces a structural change from a random coil structure to a β-sheet resulting in a transformation from low n oligomers to higher order structures (2), and finally to ThT positive fibrils and fibers (3 and 4). The MQ extract can induce the formation of different types of aggregated species that are ThT negative (5), implying a change in the β-sheet structure conformation. A proposed mechanism is that the MQ extract destabilizes the β-sheets by interacting with the aromatic rings of polyphenols and with aromatic rings of the key residues in Aβ. These interactions could favor the formation of unstructured non-toxic aggregates as seen in our results.

and help prevent the appearance of AD. The use of this extract proposes a new developmental strategy in the search for therapies or nutraceuticals as prophylactics for cognitive deterioration.

In conclusion, the MQ extract could be useful in neuronal protection against Aβ toxicity. This prevention is related with the capacity of the extract to alter the aggregation kinetics of the peptide, generating larger aggregates with less toxic capacity. These results suggest the possibility that the MQ extract could be a good nutraceutical complement to the classical therapy.

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