Neuroprotective effects of Elephant Black Garlic extract (*Allium ampeloprasum*) against β-amyloid toxicity on hippocampal slices

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ABSTRACT

It is widely known that the toxic effects of the beta-amyloid soluble oligomers (SO-Aβ) are central elements on the pathogenesis of the Alzheimer Disease (AD). In our group, it has been demonstrated that SO-Aβ has the capacity to induce the pore formation in the plasma membrane wich generates an influx of calcium and leakage of big molecules, like ATP. Mainly, the cytosolic Ca^{2+} overload, induces mitochondrial dysfunction and synaptic failure, leading to cellular death. Several evidence suggests that common black garlic (*Allium sativum*) has abeneficial effects on the Aβ toxicity, and these effects that are proposed could be mediated by S-allyl-cysteine (SAC), the main organosulfur metabolite present in the garlic. *Allium ampeloprasum* is an endemic species from the Chiloé Island, located in the Northwestern Patagonia of Chile, the study of the Elephant Black Garlic Extract (BG) has recently started in our lab, and the chemical composition and the neuromodulatory properties are the main objective of this research. BG (10 µg/mL) induces a neuroprotective effect evaluated by viability assays (MTT), recovers the cell viability about 52±5% on PC12 cell treated with Aβ during 24h. Additionally, the synaptic activity measured by Ca^{2+} oscillation on hippocampal neurons, shows that BG recovered the frequency of Ca^{2+} oscillations of the neurons previously treated with Aβ during 24 h, about in a 54±6%. To verify these effects on more physiological model, we used hippocampal slices to tests the effects of BG on slices treated acutely (3h) with Aβ (2.5 µM). We observed that the slice viability was maintained near to control conditions when the BG was co-incubated with Aβ (Aβ: 53±5%; Aβ+BG: 113±6%). In parallel, the mitochondrial functionality was measured on hippocampal slices using JC-1 dye and fluorescence techniques. The presence of Aβ induced a strong fall in the mitochondrial potential near to 36±7%, while the co-incubation with BG maintains a potential with values near to control conditions. These last observations where correlated with changes on the key proteins related with mitochondrial dynamics in hippocampal slices [(Mfn1, Aβ: 48±7%; Aβ+BG: 79±9%) (DRP1, Aβ: 118±15%; Aβ+BG: 103±14%)]. **Our results suggest that BLACK ELEPHANT GARLIC MELIMEI can induce a strong protection of the neuronal network against Aβ toxicity and could represent an interesting source of new compounds that can be useful to interfere with the physiopathology of amyloid beta peptide oligomers**.

RESULTS

Comparative chemical composition of white and black garlic from *A. ampeloprasum*

Compound	Retention time (t_R) (min)	White Garlic Area (Average)	White Garlic Area (%)	Black Garlic Area (Average)	Black Garlic Area (%)	% Area BG/ % Area WG
Diallyl sulfide	5,297	3,73E+05	0.37	9,36E+04	17.46	47.13
Methyl allyl disulfide	6,581	5,23E+05	0.52	-	-	-
3H-1,2-Dithiole	7,538	4,22E+06	4.19	1,22E+05	22.86	5.45
Diallyl disulphide	10,526	8,40E+07	83.42	1,59E+05	29.74	0.36
1-Allyl-2-isopropyldisulfane	10,758	1,75E+06	1.74	-	-	-
(E)-1-Allyl-2-(prop-1-en-1-yl) disulfane	10,905	3,54E+06	3.51	-	-	-
Trisulfide, methyl 2-propenyl	11,694	3,62E+05	0.36	2,31E+03	0.43	1.20
4-Methyl-1,2,3-trithiolane	12,041	5,85E+05	0.58	8,63E+04	16.10	27.69
3-Vinyl-1,2-dithiacyclohex-4-ene	12,788	2,14E+06	2.13	1,71E+04	3.19	1.50
4H-1,2,3-Trithiine	13,009	5,27E+05	0.52	1,62E+04	3.03	5.78
2-Vinyl-4H-1,3-dithiine	13,272	1,86E+05	0.18		-	-
Trisulfide, di-2-propenyl	15,082	8,04E+05	0.80	1,50E+04	2.79	3.50
1-Allyl-3-propyltrisulfane	15,314	1,43E+05	0.14	-	-	-
(E)-1-Allyl-3-(prop-1-en-1-yl) trisulfane	15,577	2,34E+05	0.23	-	-	-
5-Methyl-1,2,3,4-tetrathiane	16,376	6,04E+05	0.60	2,36E+04	4.40	7.34
1,3-Dithiole-2-thione	16,713	2,39E+05	0.24	-	-	-

Table 1. Chemical profile of sulfur compounds of white and black garlic extracts from *Allium ampeloprasum*

Antioxidant capacity of a black garlic extract from *Allium ampeloprasum*

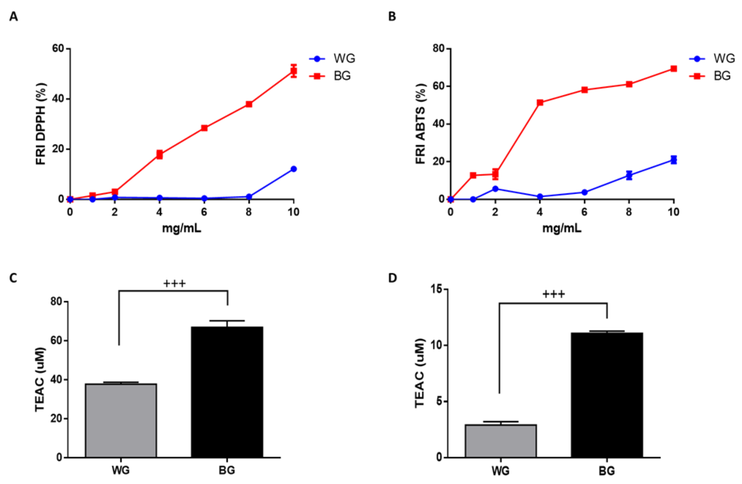


Figure 1. Antioxidant activity of WG and BG extracts. A) Inhibition of the free radical DPPH elicitid by a range of BG and WG extracts concentration (0-10 mg/mL). **B)** Inhibition of the free radical ABTS elicited by the same experimental conditions used in A. **C)** and **D)** Comparisson of the antioxidant capacity of WG and BG at 10 mg/mL taked from panles A and B, respectively. FRI: Free Radical Inhibition. (n=3; N=9) $^{+++}p<0.001$ WG vs BG.

Neuroprotective effects of BG on chronic cellular models of SO-Aβ toxicity

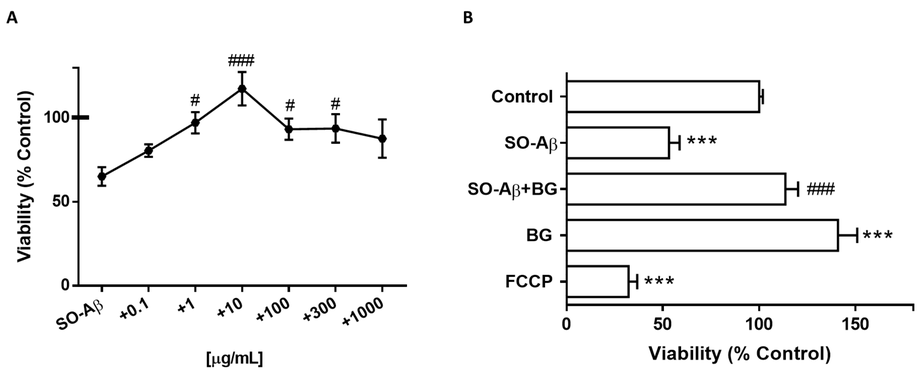


Figure 2. BG prevents the decrease of cellular viability induced by SO-Aβ. A: PC-12 cells were incubated with SOAβ (0.5 µM) and BG (0.1 to 1000 µg/ml) for 24 h, to measure cell viability. **B:** Hippocampal slices were incubated for 3 h with SO-Aβ (2.5 µM), BG (20 µg/mL) and FCCP (10 µM). The values are expressed as a percentage of the control (n=3, N=9), *vs Control and # vs SO-Aβ.

BG prevents mitochondrial dysfunction induced by SO-Aβ

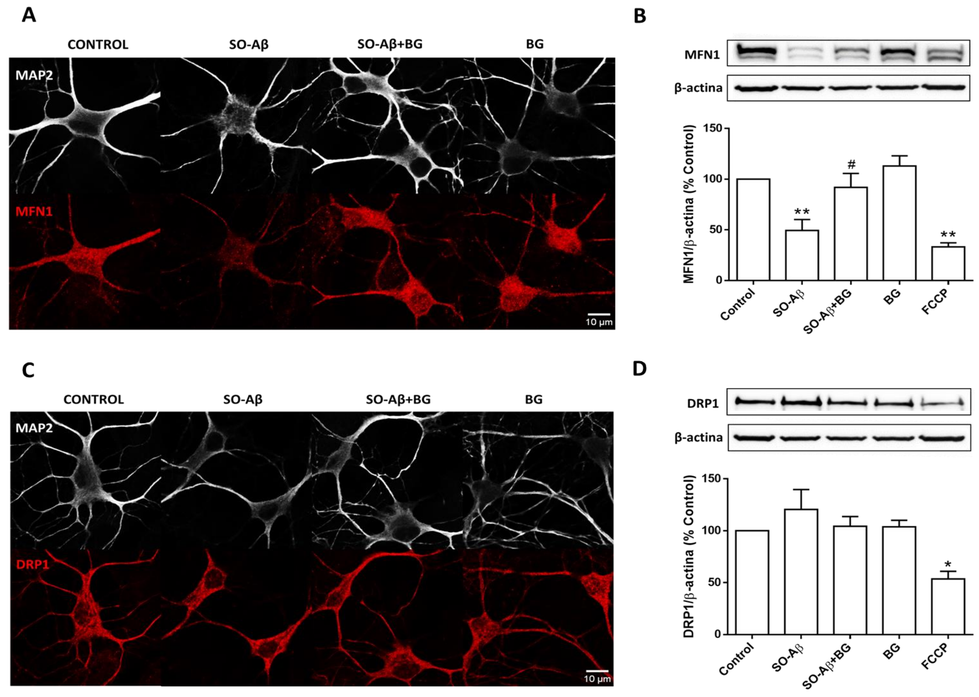


Figure 3. Effects of BG and SO-Aβ on the expression levels of mitochondrial dynamics proteins MFN1 and DRP1. A: Images of confocal microscopy of hippocampal neurons (10 DIV) treated for 24 h with SO-Aβ (0.5 µM), BG (10 µg/ml) and SO-Aβ+BG. The image shows the MAP2 neuronal marker (white) and the MFN1 protein (red) (n=3; N=15). **B:** Western blot of MFN1 using a specific antibody (upper panel) in hippocampal slices treated for 3 h with SO-Aβ (2.5 µM), BG (20 µg/ml), SO-Aβ+BG and FCCP (10 µM). The quantification of the intensity of the bands is represented as a percentage of the control without treatment in the graph (n=10). **C:** Neurons treated with the same conditions as A to measure the immunureactivity of the DRP1 protein (n=3; N=15). **D:** Western blot to measure DRP1 protein levels, using the same conditions as in B (n=10). * vs control and # vs SO-Aβ.

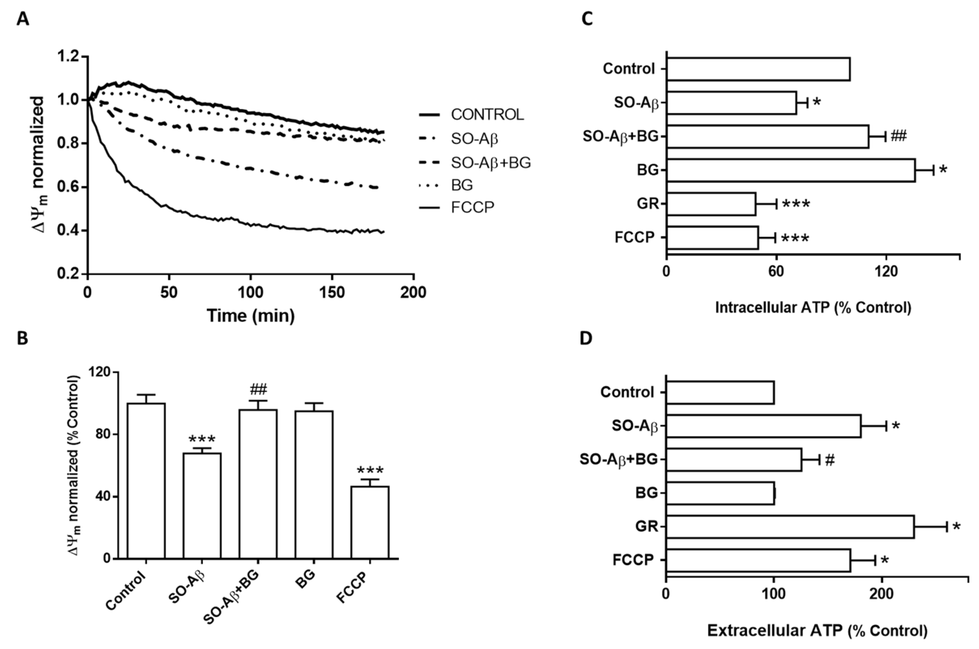
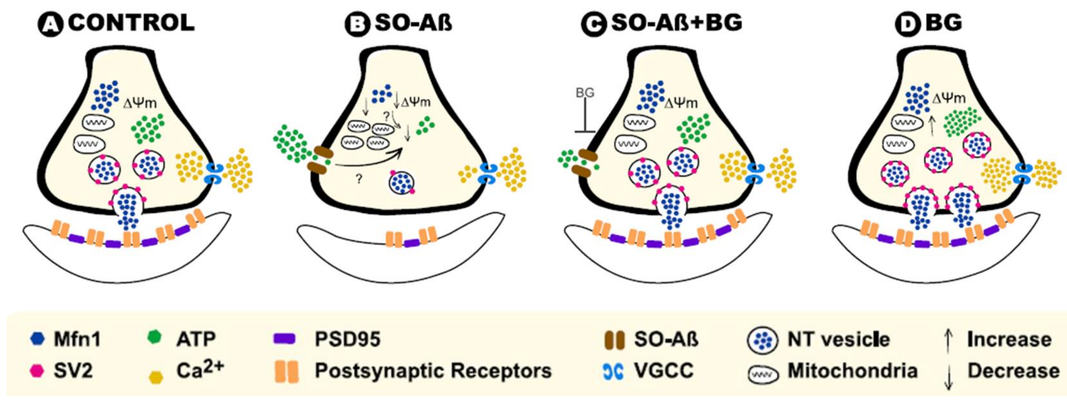


Figure 4. Effects of BG on the decrease in mitochondrial membrane potential (ΔΨm) and ATP levels produced by SO-Aβ. A: Temporary in vivo course of ΔΨm in hippocampal slices treated with SO-Aβ (2.5 µM), BG (20 µg/ml), SO-Aβ+BG and FCCP (10 µM). **B:** Quantitative analysis of the end point of the kinetics of A as an indicator of decrease in ΔΨm (n=5; N=16). **C:** Slices were treated for 3 h with SO-Aβ (2.5 µM), BG (20 µg/ml) and SO-Aβ+BG to measure intracellular ATP levels. **D:** Quantification of extracellular ATP in the supernatant of the hippocampal slices of A. Gramicidin (100 µg/ml) and FCCP (10 µM) were used as positive controls of cellular toxicity. The values are expressed as a percentage with respect to the control (n=6). * vs control and # vs SO-Aβ.

CONCLUSIONS

Our results suggest that elephant black garlic MELIMEI extract exerts a strong protection of the neural network against toxicity induced by SO-Aβ, by regulating mitochondrial machinery and enhancing synaptic activity. Therefore, the sulfur compounds present in elephant black garlic could be useful in the development of new pharmacological tools that contribute to prevent or treat AD in early stages



METHODS

Antioxidant activity: The activity of the stable DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical, was determined using the method described by Brand-Williams et al. (1995). To estimation of TEAC Activity was used the ABTS•+ Assay (Ren, Wang et al. 1999). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard to construct the calibration curve. The results were expressed as Trolox equivalent antioxidant capacity values (TEAC) (Nenadis, Wang et al. 2004).

Hippocampal slices: Brains of C57BL/6 mice (3-4 months old) were mounted on a Microslicer. The hippocampi were isolated and deposited in an aCSF solution (34°C, 1 h, 95% O_2/5% CO_2).

Mice primary hippocampal culture: 18E embryo hippocampus (C57BL/6J) were plated at 320,000 cells/ml. Cells were incubated as described in Gavilán *et al.* 2018, and used at 10 DIV.

$Aβ_{1-40}$ peptide: Stock was reconstituted in DMSO. To obtain SO-Aβ, the peptide was aggregated in PBS 1X at 80 µM (500 rpm, RT, 4 h).

Cell viability: Slices were incubated in MTT (0.5 mg/mL, 1 h, 37°C). Insoluble formazan was solubilized in 100 µl of 2-propanol, and the absorbance was read (570 nm) in a multiplate reader.

Mitocondrial membrane potencial (ΔΨm) : Slices were incubated with the JC-1 probe (2 µM, 30 min, 37 °C). The fluorescence were captured in a multiplate reader. The 590/520 nm ratio indicates the variations in the ΔΨm.

ATP levels: Slices and supernatants were incubated with ATP determination KIT as described in Fuentealba *et al.* 2011. The luminescence was detected in a multiplate reader (28 °C, 560 nm).

Immunocytochemistry: The cells were fixed, permeabilized and blocked (4% PFA, 0.1% Triton X-100, 10% HS). Samples were incubated with primary and secondary antibodies. Dako mounting medium was used. Images (60x) were acquired using LSM780 NLO (confocal) microscope; its processing and quantification was made with ImageJ.

Western blot: Samples were denatured (100°C, 10 min), subjected to SDS-PAGE, and transferred to PVDF membranes, which were blocked with 5% non-fat milk in TBS-T and incubated with primary (ON) and HRP-conjugated secondary antibodies (1 h). Immunoreactive bands were exposed using Clarity Western ECL Substrate and an Odyssey FC detection system. Image analysis was done with the ImageStudio software.

Stadistical analysis: Data is presented as the mean ± SEM and expressed as a percentage with respect to the control without treatment. Statistical significance was determined using one-way ANOVA, using the GraphPadPrism

STATEMENT

Allium Ampeloprasum is a different species than common garlic (allium sativum). This particular allium is only produced in Chiloé Island, northwestern Patagonia.

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