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Aloe arborescens Extract Protects IMR-32 Cells against Alzheimer Amyloid Beta Peptide via Inhibition of Radical Peroxide Production

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Aloe arborescens is commonly used as a pharmaceutical ingredient for its effect in burn treatment and ability to increase skin wound healing properties. Besides, it is well known to have beneficial phytotherapeutic, anticancer, and radio-protective properties. In this study, we first provided evidence that *A. arborescens* extract protects IMR32, a neuroblastoma human cellular line, from toxicity induced by beta amyloid, the peptide responsible for Alzheimer's disease. In particular, pretreatment with *A. arborescens* maintains an elevated cell viability and exerts a protective effect on mitochondrial functionality, as evidenced by oxygen consumption experiments. The protective mechanism exerted by *A. arborescens* seems be related to lowering of oxidative potential of the cells, as demonstrated by the ROS measurement compared with the results obtained in the presence of amyloid beta (1-42) peptide alone. Based on these preliminary observations we suggest that use of *A. arborescens* extract could be developed as agents for the management of AD.

Keywords: *Aloe arborescens*, Beta amyloid peptide, Radical oxygen species (ROS).

Alzheimer's disease (AD), one of the most common neurodegenerative disorders, is characterized by a protein misfolding disease due to the accumulation of abnormally folded beta-amyloid protein, a peptide of 1-42 aminoacids [1]. This neurodegenerative disorder of the brain is chronic and progressive and is also characterized clinically by deterioration in the key symptoms of behavioural and cognitive abilities. Oxidative stress has been strongly implicated in the patho-physiology of this neurodegenerative disorder [2,3]. Central neurons are especially vulnerable to insults induced by oxidative stress, due to their higher levels of polyunsaturated fatty acids and lower levels of brain-resident antioxidants, as well as high oxygen consumption. ROS, produced by damaged mitochondria during oxidative stress [4], can damage proteins, nucleic acids, and membrane polyunsaturated fatty acids, causing lipid peroxidation and leading to loss of membrane integrity, and increasing permeability to Ca^{2+} in the plasma membrane [5]. In this light, plants and their extracts, appreciated for their specific aroma, nutraceutical, and therapeutic properties such as antimicrobial, antiproliferative, anti-inflammatory, immunostimulant, and antioxidative [6], have been suggested as possible agents for the prevention of cellular damage in neurodegenerative disorders [7,8]. Recent studies have demonstrated that *Aloe arborescens*, one of the main species of *Aloe* used worldwide, had immunostimulating activity in animal trials [9,10], and it was found to have beneficial phytotherapeutic and anticancer properties [11]. The aim of the present study was to analyse the effect of *A. arborescens* extract preconditioning on cellular vitality, oxidation potential and cellular respiration in IMR32 cells treated with beta-amyloid peptide (AβP). To investigate the neuroprotective effects of *A. arborescens* pretreatment on neurons insulted by Aβ (APB), the peptide responsible for Alzheimer's disease, we used the MTS reduction assay on neuroblastoma differentiated human cells (IMR32) treated with beta-amyloid, a 1-42 peptide, responsible for Alzheimer's disease. As shown (Figure 1), IMR-32 cells pre-incubated for 48 h with *A. arborescens* extract (0.1 mg/mL) and successively treated for 24 h with Aβ (10 μM) peptides, showed an increased cell viability compared with cells treated with a beta-amyloid peptides alone. In fact the pretreatments preserved the neurons by a reduction of cellular availability of 60% determined by Aβ peptide maintaining values approximately similar to the control. *A. arborescens* extract alone did not affect the cellular availability compared with untreated controls.

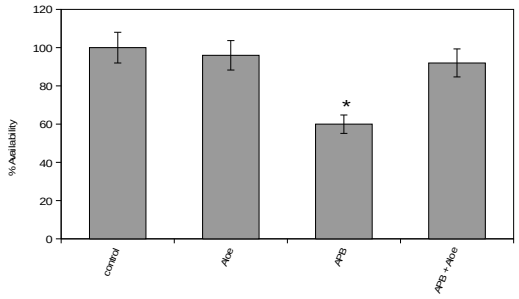

Figure 1: Effects of *Aloe arborescens* extract pretreatment (for 48 h at 0.1 mg/mL) on IMR-32 cells after 24 h of incubation with 10 μM beta-amyloid peptides (APB). The cell survival is expressed as percent of cells untreated. Cells (10,000 cells/well) were cultured with substances under analysis (experimental conditions are reported in Experimental), and the availability of cells was measured by MTS assay. All values indicate means ± S.E. of eight independent experiments. Significantly different from untreated cells: $*P < 0.01$.

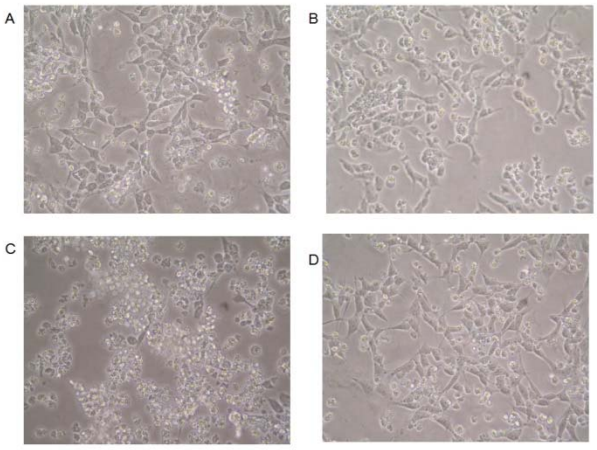

Figure 2: Phase-contrast micrography showing IMR-32 cells in medium (panel A); treated with 0.1 mg/mL of *Aloe arborescens* extract (panel B); treated with 10 μM beta amyloid peptides (APB) (panel C); and pre-incubated for 48 h with *A. arborescens* before treatments with APB (panel D).

The effect of *A. arborescens* extract on IMR32 treated with beta-amyloid peptide (APB) was evaluated also morphologically by phase-contrast microscopy (Figure 2). Untreated IMR32 (Figure 2A), cells treated with *A. arborescens* extract alone (Figure 2B) and 24 before the treatment with beta-amyloid (Fig 2D) show an intact cell surface with typical cytoplasmic extensions. Differently, IMR32 cells treated with beta amyloid peptide (Figure 2C) show evident signs of cellular suffering with an elevated number of neurons shrunken with condensed nuclei.

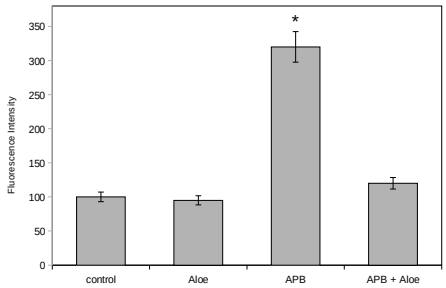

Figure 3: Effect of *Aloe arborescens* extract on ROS production (expressed as Fluorescence Intensity) in IMR32 cells treated with beta-amyloid peptide (APB). Experimental details are reported in Experimental. All values indicate means ± S.E. of eight independent experiments: significantly different from untreated cells: **P* < 0.01.

Since oxidative stress has been strongly implicated in the patho-physiology of the neurodegenerative disorder by beta amyloid peptide we hypothesized that the protective effect by *A. arborescens* may be due to a control exerted on radical oxygen production. In this regard, the ROS measurement is reported for IMR32 in different experimental conditions. It is evident (Figure 3) that exposure of neuroblastoma cells to beta-amyloid peptide increased production of ROS, whereas previous treatment with *A. arborescens* extract defends the cells by oxidation processes showing ROS values similar to the control. Since ROS-mediated damage to biomolecules can have direct effects on the components of the electron transport system, here we examined the respiration rate on intact cells in the different experimental conditions. Hence, basal respiration of IMR32 treated with *A. arborescens* and APB was measured in intact cells suspended in a medium containing glucose (Fig 4). Basal respiration rates (Routine – Fig 4A) reflect mitochondrial respiration fuelled by NADH and FADH2 generated from the TCA cycle after decarboxylation of pyruvate, which was

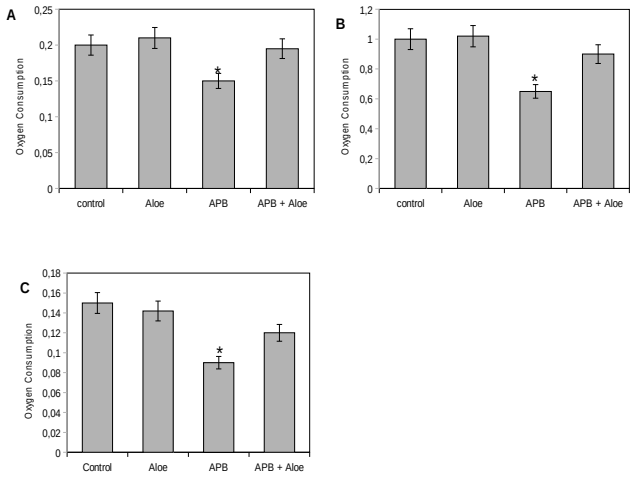

Figure 4: Oxygen Flux (expressed as pmol.s^{-1}.μg^{-1} protein) in intact IMR32 (control), treated with *Aloe arborescens* and with beta-amyloid peptide (APB). (A) Basal Respiration (Routine); (B) Uncoupled respiration and (C) Leak respiration. Details are reported in Experimental section. All values indicate means ± S.E. of eight independent experiments: significantly different from untreated cells: **P* < 0.01.

produced in the cytoplasm from the conversion of glucose in the media. As is evident, the measurement of oxygen consumption demonstrates a decrease in the cells treated with APB (according to literature data [12]) and a maintenance of normal O_2 flux in the IMR32 pretreated with *A. arborescens* with respect to the control. Then, in order to assay the maximum respiratory capacity of non-permeabilized cells, the mitochondrial electron transport system was uncoupled by the addition of FCCP (Figure 4B). The observed respiration rates were six-fold higher than basal respiration, maintaining, however, the difference evidenced in the Routine state. Finally, leak respiration was measured in the absence of FCCP after addition of oligomycin to block F0F1-ATP synthase (Fig 4C). Among the three assessed respiration parameters, leak respiration accounted for the lowest respiration rates measured. So, also in this case, APB treatment significantly altered cellular respiration compared with the control and the pretreatment with *A. arborescens* extract preserved this effect.

In conclusion, this study, for the first time, showed that *A. arborescens* extract preconditioning, enhances neuron resistance to toxicity induced by beta-amyloid treatment, increasing significantly the cellular availability. The molecular mechanism for the basis of this observed phenomenon seems to be a link to a control of oxidative cellular potential, according to previous data [13]. In fact IMR32 cells treated with *A. arborescens* 24 h before treatment with beta-amyloid, maintain low levels of reactive oxygen species (ROS), unlike cells treated with Aβ peptides where the formation of ROS is considerable and plays a significant role in effecting cellular pathogenesis.

Experimental

Cell culture: Human neuroblastoma IMR-32 cells were grown in minimum essential medium supplemented with 10% heat inactivated fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin and cultured at 37 °C in an atmosphere of 5% CO_2 in air. After a week, the differentiated cells were plated at an appropriate density, according to each experimental procedure. Aβ peptide was obtained from Peptide Speciality Laboratories GmbH (Heidelberg, Germany). Stock solution of Aβ peptide were prepared according to the manufacturer's instructions and stored at −80 °C.

Preparation of leaf extract: *Aloe arborescens* Miller specimens leaves were collected from 3 year old plants grown in greenhouses at Tuscia University, Viterbo (Italy). After several washings in distilled water, the spines were excised from leaves with a razor blade; epidermis tissue was separated from the leaf gel, weighed, homogenized with an equal volume (w/v) of absolute ethanol and maintained for 20 min in the dark at room temperature. The homogenate was then centrifuged at 2000 rpm for 5 min at room temperature, the pellet discarded and the supernatant dried in a nitrogen flow. The resulting residue was diluted with phosphate buffered saline (PBS, pH 7.4) at concentrations of 20 mg/mL, centrifuged at 7700×*g* for 15 min, and filtered through a 0.22-μm filter. The sterile extract was employed for treatments at 0.1 mg/mL in cell culture medium (preliminary studies were performed to define the most appropriate experimental dose based on cell survival and preserved morphology) 48 h before the treatments with beta-amyloid peptides, which was added to each experimental set at a final concentration of 10 μM. Control experiments (untreated cells) and ethanol treated cells showed no significant differences (data not shown).

Direct toxicity study: For determination of viability, IMR-32 cells were plated in 96-well plates at a density of 10,000 cells/well and incubated, after pretreatments with *A. arborescens* extract (0.1 mg/mL), in the presence of 10 μM Aβ peptides. Cell survival was evaluated after 48 h by MTS reduction assay. The MTS assay is a sensitive measurement of the normal metabolic status of cells: the intracellular soluble formazan produced by cellular reduction of the MTS was determined by recording the absorbance of each 96-well plate using the automatic microplate photometer at a wavelength of 490 nm. The morphological features of cells observable after 48 h of incubation with the different treatments were analyzed and photographed by phase-contrast microscopy 40×.

Detection of ROS: The detection of ROS was performed after staining of cells with DCFDA Cellular ROS Detection Assay Kit. Briefly, cells treated in different experimental conditions, were grown in 96-well microplates with 25,000 cells per well and treated successively with 2',7'–dichlorofluorescein diacetate (DCFDA), which is initially non-fluorescent and is converted by oxidation to the fluorescent molecular DCF. DCF was then quantified using a CytoFluor Multi-well Plate Reader, with 485 nm excitation and 538 nm emission filters.

Respiration of intact cells: A particular advantage of studying intact cells is the quantification of respiration in the physiologically controlled state. Cellular basal oxygen consumption (Routine respiration) was measured at 37°C using a Clark-type oxygen electrode (Strathkelvin Instr., Glasgow, UK) under continuous stirring. Five million cells were added to 2 mL respiration medium containing 120 mM NaCl, 15 mM glucose, 3.5 mM KCl, 2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 1.2 mM Na_2SO_4, and 0.4 mM KH_2PO_4 (pH 7.2). Excess capacity for electron entry was determined by uncoupling the cells with 0.5 μM FCCP. Proton leak was measured with addition of olygomycin (2 μg/mL) in the absence of FCCP.

Statistical analysis: The data were analyzed by one-way ANOVA, followed by *post hoc* Newman–Keul test for multiple comparisons among group means, using a Prism TM computer program (Graph-Pad, San Diego, CA, USA), and differences were considered statistically significant if $P < 0.01$. All results are presented as the mean ± S.E.M. of at least 7 different experiments.

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