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In vitro effects of serotonin, melatonin and other related indole compounds on amyloid-*β* kinetics and neuroprotection

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Abbreviations: **3IA**, 3-indoleacetic acid; **A*β***, Amyloid-β peptide; **AD**, Alzheimer´s Disease; **CD**, Circular Dichroism; **HO**, Heme Oxygenase; **Hsp**, heat shock protein; **Mel**, melatonin; **MTT**, Thiazolyl Blue Tetrazolium Bromide; **Nser**, N-acetylserotonin; **NPT**, without pretreatment; **Nrf2**: nuclear factor-erythroid 2; **PC12**, pheochromocytoma cells; **Phol**, tryptophol; **PT,** pretreatment; **qPCR,** quantitative PCR; **Ser**, Serotonin; **Tee**, tryptophan ethyl ester; **TEM**, Transmission Electron Microscopy; **ThT**, Thioflavin T; **Trpa**, tryptamine; **Tryp**, tryptophan; **ROS**, Reactive Oxygen Species.

Keywords: amyloid-β, fibril formation, indolic, neuroprotection, vitagene system

Abstract

Scope: Amyloid-β peptide (Aβ) is the main component of senile plaques in Alzheimer´s disease (AD). The inhibition of Aβ assembly, the destabilisation of Aβ aggregates and the decrease of its cytotoxicity for the prevention of neuronal death are considered neuroprotective effects. In this work, the protective effects against Aβ aggregation and cytotoxicity of eight indolic compounds are evaluated: tryptophan, tryptamine, serotonin, tryptophol, N-acetylserotonin, 3-indoleacetic acid, tryptophan ethyl ester and melatonin.

Methods and results: Thioflavin T spectroscopic assay, Transmission Electron Microscopy, western blotting, Circular Dichroism, NMR, cell viability (thiazolylblue tetrazolium bromide assay), quantitative PCR, and heme oxygenase activity are used. Serotonin is the most effective compound for inhibiting amyloid-β peptide aggregation. Almost all the indolic compounds tested prevent amyloid-β peptide-induced and increase cell viability, being between 9 and 25%. Melatonin and Serotonin are the most active. Moreover, Serotonin increased the expression of SIRT-1 and 2, heat shock protein 70 and hemeoxygenase activity, this being a possible mechanism underlying the observed neuroprotective effect.

Conclusion: Melatonin and other related indolic compounds, mainly serotonin, show an inhibitory and destabilizing effect on amyloid-β peptide fibril formation and they possess neuroprotective properties related to the vitagenes system.

1 Introduction

Alzheimer´s disease (AD) is the most common neurodegenerative disorder defined by a progressive decline in memory and other cognitive functions [1]. This disease is characterized by the deposition of extracellular Amyloid-β (Aβ) peptide in the brain, leading to the formation of amyloid plaques, as well as by intracellular hyperphosphorylation of τ protein forming neurofibrillary tangles [2]. Currently, the aggregation of Aβ into toxic protofibrils is considered the main key pathogenic event in the onset of AD [3]. For this reason the strategy of inhibiting Aβ aggregation appears to be a valid disease-modifying therapy for AD [4].

Indole-derived compounds are present in significant quantities in food. Recently, a revision work has reported their concentrations in fermented foods [5]. The appearance of these compounds has been related to yeast metabolism [6]. Indeed, tryptophol (Phol) is present in beer in concentrations of 0.242 mg/L [7] and serotonin (Ser) has been determined in fermented products such as wines and beer in higher concentrations ranging from 2.94 to 24.2 mgL^{-1} [8, 9].

Melatonin (Mel) is a neurohormone secreted by the pineal gland. It is synthesized from the essential dietary amino acid tryptophan (Tryp) through the Ser pathway (Figure 1A). This molecule is involved in many physiological processes such as circadian rhythm, antioxidant activity and regulation of the immune system [10-12]. Moreover, several healthy properties have been attributed to this bioactive [13]. Concentrations of Mel vary according to age. With the process of aging, a decline of several precursors of Mel

including Tryp and Ser occurs and their reduction may be linked to the appearance of AD [14, 15]. Recently, the neuroprotective effect of Mel against Aβ aggregation has been demonstrated [16]. Moreover, another work suggests a possible role of the indole ring in the inhibitory properties of indole derivatives [17].

Emerging evidence shows the high potential of the vitagene system as a target for neuroprotective strategies. Vitagenes are a group of genes involved in preserving cellular homeostasis under stressful conditions. The heat shock proteins (Hsp) (hemeoxygenase) HO-1/Hsp32, Hsp70 by the thioredoxin system and sirtuin proteins form the vitagene family [18, 19]. Moreover, dietary antioxidants, such as polyphenols (curcumin and resveratrol) have recently been demonstrated to be neuroprotective through the activation of hormetic pathways, including vitagenes [20].

This study focuses on the protective properties of Mel and other indole-related compounds involved in its synthesis against Aβ aggregation and toxicity. For this purpose we have used different techniques: hioflavin T (ThT) essay, cell viability (thiazolyl blue tetrazolium bromide [MTT]), western blotting, Transmission Electron Microscopy (TEM), Circular Dichroism (CD), HPLC, NMR, quantitative PCR (qPCR) and HO activity.

2 Material and methods

2.1 Chemicals and standards

Tryptamine (Trpa), Ser, Phol, N-acetylserotonin (Nser), Mel, ThT, DMSO, DMEM-Glutamax, trypsine-EDTA, MTT, PBS, L-glutamine, fetal horse and fetal bovine serum, streptomycin, tween 20, D (+)-sucrose, Tris-HCl, leupeptin hemisulphate, EDTA, β-NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, biliverdin reductase, hemin and SIRT-1, SIRT-2, Hsp70, HO-1 and β-actin primers, were purchased from Sigma Aldrich (Steinheim, Germany). 3-indoleacetic acid (3IA) and uranyl acetate dihydrate were purchased from Fluka Biochemika (Steinheim, Germany). $A\beta_{1-42}$ peptide was provided by Alexotech (Umeå, Sweden). Na_2HPO_4/NaH_2PO_4 and NaCl were supplied by Panreac (Barcelona, Spain). Pheochromocytoma (PC12) cells were supplied by American Type Culture Collection (Manassas, USA). Aβ N-terminal specific antibody 82E1 was obtained from IBL (Minneapolis, Minnesota). Aβ (1-16) monoclonal antibody 6E10 was purchased from Covance (Japan). Anti-mouse IgG, HRP-linked antibody was supplied by Cell Signaling Technology (Danvers, Massachusetts). HyperPAGE prestained protein marker, TRIsure reagent and SensiFAST ™ SYBR® No-ROX Kit were purchased from Bioline (London, UK). L-tryp, SDS-Solution 10%, glycine, bromophenol blue and glycerol anhydrous were purchased from Applichem (Darmstadt, Deutschland). Pefabloc®SC (AEBSF) and pepstatin were obtained by Roche (Basel, Switzerland). 2-Mercaptoetanol, 10× Tris/glycine/SDS (10× premixed electrophoresis buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3); 10× Tris/glycine (10× premixed electrophoresis buffer), 4-20% Mini-PROTEAN® TGX Stain-Free gel, 15-well precast polyacrylamide gel (8.6 × 6.7 cm) and Immun-Blot olyvinylidene fluoride

(PVDF) membrane were purchased from Bio-Rad (München, Germany). ECL 2 Plus western blotting substrate and RevertAid First Strand cDNA Synthesis Kit were sourced from Thermo Scientific (Rockford, USA). Carbon-coated grids (300 mesh, copper) were purchased from EMS (Hatfield, United States).

2.2 Measurement of Aβ aggregation and destabilisation using ThT assay

The process of aggregation was monitored using the ThT assay. ThT is able to assemble with misfolding proteins and emit fluorescence as a result of this union [21]. A stock solution of 221 µM of $A\beta_{1-42}$ protein was dissolved in buffer (Na_2HPO_4/NaH_2PO_4 [25 mM] pH 7.4, 140 mM NaCl) and a stock solution of each indole compound was prepared at 50 mM in DMSO.

With regard to the aggregation assay, $A\beta_{1-42}$ (10 µM final concentration) was mixed in equal volumes with each compound (100 µM final concentration) and ThT (25 µM final concentration) on black 96-well plates. ThT fluorescence was monitored using a multi-detector microplate reader (Synergy HT, Biotek®) fluorescence spectrophotometer set at 450 nm for excitation and 485 nm for emission wavelengths. Fluorescence emission data were recorded every 2 hours over a 48-h period. At the end of the experiment, samples were frozen at -80°C. These samples were later used for electrophoresis and western blot purposes and TEM analysis. The same protocol was used for the disaggregation assay, with the only difference that the $A\beta_{1-42}$ protein was previously fibrillated during 3 d at 37°C with continuous agitation (400 rpm).

2.3 Electrophoresis and western blot analysis

15 µL of samples (after inhibition and destabilisation assays) were diluted with 5 µL of Tris-Base (0.125 M), glycerol (20%, v/v), SDS (4%), 2-β-mercaptoetanol (10%, v/v) and bromophenol blue (0.06%, w/v) buffer. After gel electrophoresis (4-20% precast polyacrylamide) proteins were transferred to PVDF membranes by applying 310 mA for at least 1 hour. The membrane was boiled for 5 minutes with PBS and then rapidly cooled. The membrane was incubated with a mix of antibodies (6E10 and 82E1. 1/6000 dilution) overnight at 4°C. The samples were then cleaned with Tris Buffered Saline with Tween and incubated with anti-mouse IgG antibody (1/10000 dilution) for 1 h. Subsequently, the membrane was cleaned with tris-buffered saline with Tween and examined by chemiluminescence (Amersham Imager 600, GE Healthcare Life Sciences).

2.4 TEM images

10 µL of samples (after inhibition and destabilisation assays) were placed on a 300-mesh carbon-coated grid and incubated for 5 minutes. The excess fluid was then removed and the grids were negatively stained for 1.5 minutes with 5 µL of a 0.5% uranyl acetate solution. Excess fluid was removed and the samples were viewed using a Zeiss Libra 120 transmission electron microscope operating at 80 kV.

2.5 Sedimentation assays

$A\beta_{1-42}$ stock solution (1 mM) was prepared by solubilizing the lyophilized Aβ peptide following a brief vortexing in sterile water at 4°C, followed by sonication for 1 minute. This peptide stock solution was aliquoted and stored at -20 °C. All subsequent steps were performed at 4°C in order to prevent any Aβ peptide polymerisation.

Ser (the most active compound) was solubilized in ethanol (stock solution of 1 mg mL^{-1}), then diluted to reach a 100 µM final concentration. Aliquots were stored at -20°C, and used the same day or during the following 2-3 days at the latest and added to a solution of phosphate buffer (10 mM final concentration, pH 7.2) and Aβ peptide (100 µM final concentration). A typical experiment was performed on a reaction mixture containing 80 µL phosphate buffer, 10 µL $A\beta_{1-42}$, and 10 µL ethanol without or with Ser; sonication was performed for 5 minutes to avoid peptide aggregation as much as possible at time t = 0 h. The solution was incubated at 15 °C for 3 d, prior to centrifugation. The Aβ solution was centrifuged at 85000 rpm for 30 min at 15°C to pellet insoluble Aβ. The pellet was discarded and the supernatant was analysed by HPLC using UV detection at 220 nm on a LC Agilent Series 1200 system (Agilent Technologies, Santa Clara, CA, USA) equipped with a Prontosil C18 column (5 µm, 250 mm x 4.6 mm), (Bischoff, Leonberg, Germany).

2.6 CD analysis

Solutions of $A\beta_{1-42}$ with Ser were prepared according to the previously-described protocol in sedimentation assay, that is, Section 2.5. CD spectra were acquired at 20°C, under an N_2 atmosphere in a quartz cell with a path length of 0.2 cm on a JASCO J-815 spectropolarimeter (Jasco, Essex, UK), equipped with a Peltier device for temperature control. Near-UV-CD spectra were measured between 200 and 250 nm at a scanning speed of 50 nm $minute^{-1}$. Data collection was repeated five times, scanning from 250 to 190 nm.

2.7 NMR analysis

NMR analysis of the interaction between $A\beta_{1-42}$ and Ser was performed in DMSO-d_6 to prevent fast Aβ aggregation in the NMR concentration range. NMR samples were prepared by dissolving the $A\beta_{1-42}$ peptide to a 0.5 mM concentration in 500 µL of DMSO-d_6; Ser was dissolved in 300 µL of DMSO-d_6 at a concentration of 60 mM. Titration experiments were performed by adding small amounts of Ser to the peptide solution. Under the experimental conditions, the self-associations of Ser and Aβ were not significant (data not shown). All 1D and 2D NMR spectra were recorded on a Bruker Avance 600 MHz spectrometer, and calibrated using TSP-d4 as an internal standard for proton chemical shifts. NMR experiments were recorded at 300 K, and data were processed using TOPSPIN software (Bruker Topspin). The sequence-specific assignment of the $A\beta_{1-42}$ was obtained using 2D total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy experiments.

2.8 PC12 cell culture and MTT assay

Rat PC12 cells were obtained from the American Type Culture Collection. They were maintained in 75 cm2 culture flasks containing 20 mL of DMEM Glutamax supplemented with 100 units mL^{-1} penicillin, 100 mg mL^{-1} streptomycin, 15% fetal horse serum and 2.5% fetal bovine serum at 37 °C with 5% CO2. Cells were passaged every 3-4 days and detached from flasks using trypsin-EDTA. Cell counts were performed using trypan blue to stain non-viable cells.

$A\beta_{1-42}$ (5 µM final concentration) and indole-derived compounds (50 µM final concentration) were diluted with serum-free culture medium and incubated with cells (at a density of 30000 cells $well^{-1}$) for 24 hours (without pretreatment [NPT] modality) or incubated for 24 h on a thermoblock with continuous agitation and then exposed to PC12 cells for 24 h (pretreatment [PT] modality) in order to screen the

neuroprotective effect of compounds. Cell viability was determined by using the MTT reduction assay. Cells were treated with 0.5 mg mL^{-1} MTT for 3 hours at 37°C. The dark blue formazan crystals that formed in intact cells were solubilized with DMSO for half an hour. The absorbance was measured at 540 nm with a microplate reader (Synergy HT, Biotek). Results were expressed as the cell viability percentage in relation to the viability of control cells (100%). All experiments were repeated at least in triplicate and performed in quadruplicate.

2.9 qPCR Analysis

Total RNA of PC12 cells after different treatments (Aβ alone/ Aβ + Ser [50 µM/ Ser 50 µM] alone) was extracted using the TRIsure reagent (Bioline) and following the manufacturer's instructions. Using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), 1 µg of the total RNA was transformed into cDNA. qPCR was performed using the SensiFAST ™ SYBR® No-ROX Kit (Bioline) and using SIRT-1, SIRT-2, Hsp70 and HO-1 primers (β-actin was used as a housekeeping gene) (Table 1). Results were calculated using the delta Ct method and represented as absolute values with arbitrary units.

2.10 HO activity

HO activity was determined by the ability to generate bilirubin from heme in the presence of biliverdin reductase and measured spectrophotometrically [22]. In brief, PC12 cells treated under different conditions (Aβ alone/ Aβ + Ser [50 µM]/ Ser [50 µM] alone) were washed with cold PBS and collected in PBS-EDTA (1 mM, pH 8) and 50 µg mL^{-1} of AEBST. Cells were disrupted by sonication and centrifuged at 105000g for 60 min at 4°C in order to obtain microsomal proteins. The protein concentration was then calculated using BSA as a standard. Microsomes (50 µg) were added to a

reaction mixture containing: biliverdin reductase (0.5 μg μL^{-1}), 1 mM β-NADPH, 2 mM glucose-6-phosphate, 1U glucose 6-phosphate dehydrogenase, 25 μM hemin, 0.25 M sucrose, 20 Mm Tris-HCl, pH 7.4. The reaction mixture was incubated at 37°C in the dark for 1 hour. The reaction was stopped by adding 0.1 mL of chloroform, after centrifugation (15000 x g), the extracted bilirubin in the chloroform layer was measured by the difference in absorbance between 463 and 530 nm and using an extinction coefficient of 40 mM cm^{-1} for bilirubin. HO activity was expressed as pmoles of bilirubin formed per milligram of microsomal protein per hour.

2.11 Statistical analysis

For cell viability measurements, SIRT-1, SIRT-2, Hsp 70 and HO-1 expression and HO activity One-way analysis of variance test ($p < 0.05$) was used [23].

3 Results

3.1 Inhibitory effects of indolic compounds on $A\beta_{1-42}$ aggregation

Eight indole-derived compounds (Figure 1A) were investigated: Tryp, Trpa, Ser, NSer, Phol, 3IA, Mel and Tee.

To determine whether indolic compounds inhibit the assembly of Aβ into filaments, ThT assay was used. ThT fluorescence is correlated to β-sheet formation and to fibril formation [21]. As illustrated in Figure 1B, the incubation of $A\beta_{1-42}$ at 37°C with continuous stirring, showed a rapid increase in the ThT fluorescence in the first hour of the experiment, the typical lag phase of aggregation process taking place in the first ten minutes of the experiment. This observation can be explained due to the use of continuous agitation conditions and the presence of salts. Comparable results

described a lag time of 3 minutes using similar experimental conditions [24]. The progressive increase in ThT fluorescence over 50 hours indicates the formation of β-sheet structures of $A\beta_{1-42}$, sufficient this time for the aggregate formation to be observed. This result was confirmed by TEM images (Figure 2B).

To evaluate the inhibitory capacity of indole-derived compounds, an initial screening was undertaken. ThT fluorescence assay was used to measure the amount of amyloid fibrils after the addition of a fixed concentration of each compound (100 µM). The screening revealed that only Ser significantly caused a dramatic reduction of aggregation (Figure 1B). Ser inhibits around 80% of the β-sheet formation at 100 µM. This result is comparable to the effect on $A\beta_{1-42}$ fibril formation of different polyphenols such as catechin and curcumin which had been previously studied [25]. In addition, the inhibitory effect of Ser at different concentrations has been examined. $A\beta_{1-42}$ was co-incubated with 10, 50 and 100 µM of Ser. Fifty and 100 µM of Ser were the active concentrations that disrupted fibril formation, presenting a significant inhibition of 30 and 80%, respectively compared to control. In contrast, at a concentration of 10 µM no inhibitory effect was observed (Figure 2A).

To confirm these results, TEM experiments were used to visualize the morphologies of $A\beta_{1-42}$ in the presence or absence of Ser. When $A\beta_{1-42}$ was incubated alone for 50 hours, aggregates were observed whereas samples containing $A\beta_{1-42}$ and 50 and 100 µM of Ser were devoid of fibrils and contained only a very small amount of aggregates (Figure 2B). The number of aggregates decreased, therefore, as the concentration of Ser increased, this being a dose-dependent effect.

To monitor the efficacy of Ser to destabilise $A\beta_{1-42}$ fibrils, different concentrations of Ser were added to pre-formed $A\beta_{1-42}$ fibrils and ThT fluorescence was measured over

48 hours. Our results have revealed that Ser destabilises pre-formed Aβ fibril in a dose-dependent manner (65, 52, and 22%, for 100, 50 and 10 µM, respectively) (Figure 3A). These results were confirmed by TEM experiments after ThT assays. TEM observation of $A\beta_{1-42}$ pre-formed fibrils shows huge amounts of Aβ fibrils (Figure 3B). When $A\beta_{1-42}$ pre-formed fibrils were co-incubated with increasing concentrations of Ser, the number of fibrils decreases.

3.2 Sedimentation assay

Sedimentation assays were performed to ensure that Ser inhibits the formation of insoluble Aβ aggregates. Aβ incubated alone or Aβ co-incubated with Ser were analysed by HPLC. Incubated solutions were previously centrifuged in order to remove insoluble aggregates and the supernatants were then analysed by HPLC. Fresh Aβ , probably monomer, was eluted at a retention time of 3.8 min as a single peak (Figure 2D). After 3 days of incubation at 15°C, the monomer peak disappeared. The chromatogram shows the presence of limited amounts of $A\beta_{1-42}$ in the soluble fractions compared to chromatogram of freshly-prepared Aβ because fibrils were spun out by centrifugation. After co-incubation with Ser, a single peak at retention time of 3.8 min was observed and attributed to soluble Aβ species (Figure 2D). This observation confirms that Ser inhibits the conversion of soluble Aβ into insoluble fibrils.

3.3 Western blot analysis.

In order to investigate whether Ser could interfere in $A\beta_{1-42}$ fibril formation, samples originating from ThT inhibition assays were used for analysis by Western Blot. 6E10

and 82E1 antibodies (reactive to amino acid residue 1-16 of beta amyloid), were used to detect selectively Aβ monomers, oligomers and fibrils with the aim of assessing the size distribution which were present in ThT samples.

With regard to the $A\beta_{1-42}$ aggregation inhibition experiment, we have analysed $A\beta_{1-42}$ alone and $A\beta_{1-42}$ with Ser at the two active concentrations (50 and 100 µM). Figure 2C showed that when the $A\beta_{1-42}$ was incubated with 100 and 50 µM of Ser, the signals are more intense in the bands corresponding to 3.5-15 KDa (monomers and dimers of Aβ) in comparison with Aβ alone. Moreover, an intense band at 125 kDa (Figure 2C) appears for Aβ alone and not for Ser at both concentrations tested, proving the fibril formation is inhibited by Ser.

3.4 CD experiments

To examine and compare conformational changes in secondary structure, the structural behavior of $A\beta_{1-42}$, with or without Ser, was monitored using CD spectrometry. Figure 2E shows the far-UV CD spectra of protein before and after 3 days of incubation with or without Ser. The freshly-prepared Aβ is an unstructured protein with a major negative peak around 200 nm, characteristic of random coil. With incubation, Aβ alone adopts a β-sheet-structure as shown by negative peaks in the 215-220 nm region which is characteristic of an aggregated protein, consistent with the literature [26, 27]. During co-incubation with Ser, Aβ remained in random coil after 3 days of incubation. Ser prevents the conformational transition from random coil to β-sheet-structure during the fibrillation process.

3.5 NMR analysis

The NMR approach was used to obtain data about the specific interaction between $A\beta_{1-42}$ and Ser. NMR analyses were performed in DMSO-d_6 to prevent fast Aβ aggregation in the NMR concentration range [28]. To monitor the interaction, titration experiments were performed by adding small amounts of Ser to the peptide solution and monitored using NMR spectra [29]. $A\beta_{1-42}$ resonances were attributed by mean of 2D-NMR experiments. The addition of Ser to a solution of Aβ induced chemical shift variations of peptide resonances (Figure 2F). Many of the proton signals of $A\beta_{1-42}$ (Val24, Asp7, Asp23 and Tyr10) shifted to a higher field, with saturable binding process indicating a direct interaction and a specific binding between Aβ and Ser (Figure 2F). Binding constants in solution were determined by fitting the chemical shift changes of amide and aromatic protons whose variations have been measured unambiguously without overlapping. Dissociation constants (Kd) were determined by curve fitting with a one-site saturation and nonspecific binding model. The dissociation constant values obtained from the chemical-shift changes of Val24, Asp7, Asp23 and Tyr10 were 1.9 ± 1.2, 5.4 ± 1.9, 0.5 ± 0.2, 4.1 ± 0.6 mM, respectively.

3.6 Neuroprotection against Aβ toxicity in PC12 cells by indolic compounds.

MTT Reduction was employed as a cell viability indicator (PC12 cells), extensively used as a quantitative and consistent colorimetric assay [30]. First, the toxicity on PC12 cells of each compound was monitored (supplementary data). Protective effects against Aβ-induced toxicity of the eight indole compounds were measured. To this aim, we tested two different treatments in order to establish the protocol to apply. On the one hand, the whole compounds were tested NPT: $A\beta_{1-42}$ (5 µM final

concentration) and compounds (50 µM final concentration) werte mixed and incubated directly with cells for 24 hours. On the other hand, with P: $A\beta_{1-42}$ (5 µM) and compounds (50 µM) were mixed and incubated for 24 hours in agitation at 37°C and then incubated with cells for 24 hours. Finally, the cell viability through MTT assay was performed.

Figure 4 illustrates cell viability expressed as a percentage relative to the untreated control cells. After exposure to $A\beta_{1-42}$ alone, viability decreased by around 50% compared with the control. Results between the two treatments show that PT protocol increased significantly ($p<0.05$; $p<0.01$) cell viability for the majority of compounds. Tryp and Tee did not present any neuroprotective effect. The increase in cell viability exhibited is ranked in the following order: Mel > Ser > Trpa > Nser > 3IA > Phol. Mel is the most active compound followed by Ser (cell viability 25% and 24%, respectively). In this case a significant increase of cell viability was observed with both protocols, augmentation being greater with PT than with NPT (cell viability 75% and 60%, respectively).

To confirm the protective effect of Ser, its effect at different concentrations (1, 5, 10 and 50 µM) against Aβ-induced toxicity was performed using PT protocol. Ser presents a dose-dependent increase of cell viability higher for high concentrations, as proved by one-way analysis of variance test, augmentation ranging between 4% to 25% ($p< 0.001$; $p< 0.0001$) (Figure 5A).

3.7. SIRT-1, SIRT2, Hsp 70 and HO-1 expression by Ser

In order to prove the mechanism involved in the neuroprotective properties of Ser, the gene expression of SIRT-1, SIRT-2, Hsp 70 and HO-1, which are genes involved in

vitagenes system, were analyzed. As Figure 5B, C and D 5C show Ser (50 µM) incubated with Aβ increased SIRT-1 (2.6 fold), SIRT-2 (2.3 fold) and Hsp70 gene expression significantly ($p<0.01$; $p<0.001$), compared to the controls (untreated cells and Aβ incubated alone). In the case of HO-1 gene expression significant differences were only observed when cells were incubated with Ser alone (Figure 5E).

3.8. HO activity by Ser

Furthermore, the microsomal HO activity in the same experimental setting described (Section 2.10) was determined. A significant increase of HO activity was observed when cells were treated with Ser and Aβ in comparison with untreated cells and Aβ (positive control). Furthermore, the incubation with Ser alone also induced a significant increase in HO activity (Figure 5F).

4 Discussion

The inhibition of aggregation or the disaggregation of Aβ oligomers and fibrils by bioactive compounds present in food and beverages are widely studied in the prevention and treatment of AD. In this context, polyphenols such as curcumin, myricetin, morin, quercetin, kaempferol (+)-catechin, (−)-epicatechin, nordihydroguaiaretic acid, tannic acid and stilbenes have been investigated extensively [31-36]. Furthermore, the role of different plant and food extracts has also been investigated, presenting satisfactory effects against Aβ toxicity [37-39].

The presence of bioactive compounds in fermented beverages has long been observed and they have been studied with great attention. Yeast transforms certain molecules such as amino acids into biologically active compounds. Among these, the

405 case of amino acid Tryp is of interest, since it is the precursor of at least three
406 biologically active compounds: Mel, Ser and Phol [6]. Mel appears in fermented
407 products in order of ng-pg mL^{-1}. However, the concentrations of Phol or Ser are higher
408 (mg L^{-1}). Their occurrence in foods and beverages contribute to a dietetic intake that
409 might exert beneficial effects [5].
410 Very few studies have probed the inhibitory effect of indole compounds on the Aβ
411 fibrillation process. Mel has been the most studied indole compound and many
412 pharmacological properties have been endorsed [13]. It shows ~~a~~ potent antioxidant
413 activity [40], promotes the synthesis of numerous endogenous antioxidant enzymes
414 [41] and improves mitochondrial energy metabolism [42].
415 With regard to the disruption or the destabilisation of Aβ oligomers and fibrils, our
416 work demonstrates through ThT assays and confirmed by TEM and Western blot
417 analysis that Ser is a potent inhibitor for both processes (an effect of 80-65%
418 respectively), leading to the formation of soluble species, as indicated by
419 sedimentation assays. These findings show similar effects reported by other
420 polyphenols of around 70-80% on the inhibition of Aβ aggregation (resveratrol, piceid
421 and rosmaric acid) [36, 43]. In contrast with our results, Phol and 3IA have been
422 reported to present an inhibitory effect using lysozyme (protein with similar
423 aggregation pattern of Aβ). However, in this work, which uses Aβ peptide as a model,
424 no effects have been observed. Moreover, and in accordance with our own work,
425 Tryp and Mel did not provide any effect on the Aβ fibrillation process [16, 17].
426 In presence of Ser the decrease in ThT fluorescence began between 20-25 hours into
427 the experiment, the moment when the elongation phase was occurring and
428 consequently oligomers and protofibrils have been formed [44]. Based on other

reports, compounds that interfere in the elongation phase but not in the lag phase, can inhibit protein assembly by blocking the interstrand hydrogen bond formation and not by stabilising the non-amyloidogenic conformations of the proteins [45]. CD experiments indicate that Ser could perturb the backbone of $A\beta_{1-42}$, inducing the remodeling of peptide assemblies into unstructured and less toxic aggregates as previously indicated [27].

Furthermore, the majority of compounds tested protect against Aβ-induced neuronal death, the most active compounds being Mel and Ser. Ser increases cell viability in the first 24 hours of Aβ fibrillogenesis (Ser + $A\beta_{1-42}$ over 24 hours with agitation [PT]) when amyloid oligomers and fibrils were already formed (Ser + $A\beta_{1-42}$ PT + 24 hours). *In vivo* results have demonstrated that the infusion of Ser directly into the hippocampus of a mouse model of AD reduced brain interstitial fluid Aβ levels by 35% over an 8-hour period compared with vehicle-treated mice. Furthermore, this work revealed the implication of Ser signalling for altering Aβ levels and plaques not only in mice but also in humans [46].

Our data shown that Mel is unable to inhibit Aβ aggregation. It is, however, the compound which presents the highest neuroprotective potential against Aβ-induced toxicity. *In vivo* studies have previously demonstrated the capacity of Mel to reduce Aβ-induced oxidative stress related to reactive oxygen species (ROS) and proinflammatory cytokines, such as IL6 and IL1-β. Moreover, Mel has exhibited protection from damage and death in brain neurons by increasing viability in hippocampal neurons and glial cells after treatment with $A\beta_{1–40}$, $A\beta_{25–40}$, and $A\beta_{1–28}$ and in murine N2a neuroblastoma and PC12 cells by using $A\beta_{25–35}$ [47, 16]. Our

results, in accordance with ref.16, show an increase of cell viability around 20-25% by Mel.

Moreover, our data shows that Ser increased SIRT-1, SIRT-2 and Hsp70 gene expression, this being a possible mechanism involved in the neuroprotection properties observed against Aβ-induced toxicity. It is known that, during AD, the down-regulation of the expression level of SIRT-1 activates NFκB, which mediates inflammatory pathway and Aβ toxicity [48,49]. Evidence for the benefits of SIRT-1 in brain aging includes the finding that increased SIRT-1 activity protects against Aβ toxicity in cell cultures and neurodegeneration in the p25/CDK5 mouse model [50]. SIRT-2, also acting via NFκB, is considered an emerging target in neurodegenerative processes as well. Additionally, SIRT2 reduces cellular oxidative stress by promoting Forkhead box O (FOXO) transactivation activity to increase the expression of the antioxidant mitochondrial superoxide dismutase and reduce the cellular ROS levels [51]. Several studies have demonstrated that the overexpression of Hsp70 inhibits the aggregation of Aβ, and protects neurons from intracellular accumulation of Aβ through promoting its clearance.

This work has demonstrated that the co-incubation of Ser cells (with or wihout Aβ) produce an increase on HO activity. However, we have not determined an increase in HO-1 mRNA expression levels. HO activity does not necessarily correlate with mRNA levels, partly because HO measures both HO-1 and HO-2 without discriminating between them [52]. HO-1 induces the nuclear factor-erythroid 2 (Nrf2), one of the most important systems that enhance cellular protection against oxidative stress. Nrf2 drives transcription of many of the genes important to free

radical scavenging, especially those involving the critical tripeptide glutathione or production of the free radical scavenger bilirubin [53].

Several studies have demonstrated that other bioactive food compounds such as resveratrol and curcumin act by modulating redox-dependent mechanisms leading to up-regulation of vitagenes in brain. Thus, resveratrol has been proved to increase HO-1 expression in PC12 cells and primary neuronal cultures, presumably through the activation of Nrf2 [54-55]. In addition, an overexpression of SIRT1 was produced by resveratrol, reducing Aβ-stimulated NFκB signaling, proving its strong neuroprotective effects in AD [56].

Furthermore, curcumin has been shown significantly to increase HO-1 in brain cells [57]. This latter effect on HO-1 can explain, at least in part, the anti-oxidant properties of curcumin, in particular keeping in mind that HO-1-derived bilirubin has the ability to scavenge both ROS and Reactive Nitrogen Species (RNS) [58].

Additionally, Mel was shown to increase the expression of SIRT1 in aged rats' dentate gyrus, suggesting a neuroprotective role of this indolic compound through the SIRT1 pathway during aging [59]. Other authors have shown that melatonin treatment effectively preserved the relative protein levels of SIRT1in the hippocampus of completely sleep-deprived rats and via a SIRT-dependent suppression of excessive inflammation and oxidative stress [60].

Our work proves that, being the most active molecule of the set of 8 indolic compounds tested, Ser has a neuroprotective effect against Aβ insult. Despite the fact that there might be other bioactive compounds present in foods in larger amounts, one of the advantages of Ser is that it is commonly found naturally in the

target organ. In addition, our RMN results prove a strong interaction between Aβ–Ser that has to be considered when evaluating the *in vivo* potential of other bioactives, due to the fact that Ser concentration may fluctuate. Additionally, it has been demonstrated that Ser increased SIRT-1, SIRT-2 and Hsp70 gene expression and HO activity, this being a possible mechanism involved in the neuroprotection properties observed against Aβ-induced toxicity.

R,H.O., T.R., M.C.G.P. and A.M.T. designed the research; R.H.O., A.B.C. and G.D.C. conducted the research; R.H.O., G.D.C., A.B.C., A.M.T., T.R., and M.C.G.P. analysed the data and wrote the paper. All the authors read and approved the final manuscript.

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The authors declare no conflict of interest.

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Figure captions

Figure 1. Inhibition of Aβ fibrils by indolic compounds (Tryp, Trpa, Ser, NSer, Phol, 3IA, Mel and Tee): (A) Tryp metabolism and intermediates involved in the synthesis of Mel; (B) Kinetics of $A\beta_{1-42}$ (10 µM) polymerisation in absence (control) and presence of indolic compounds (100 µM) measured by ThT assay.

Figure 2. Inhibition of Aβ fibrils by Ser: (A) kinetics of $A\beta_{1-42}$ (10 µM) polymerisation in presence of Ser (10, 50 and 100 µM) measured by ThT assays; (B) TEM observation of $A\beta_{1-42}$ in absence (left) or in presence (right) of 50 and 100 µM Ser after 50 h incubation; (C) Effects of Ser at 50 and 100 µM on the inhibition of Aβ fibrils tested by Western-blot; (D) Sedimentation assays in the absence and presence of Ser (Ser to $A\beta_{1-42}$ ratio 1:1); (E) CD spectra of $A\beta_{1-42}$ in the absence and presence of Ser (Ser to $A\beta_{1-42}$ ratio 1:1); (F) NMR spectra (left) and proton chemical shift changes (right) of $A\beta_{1-42}$ in the absence and presence of different concentrations of Ser.

Figure 3. Destabilisation of Aβ fibrils by indolic compounds: (A) Kinetics of pre-formed $A\beta_{1-42}$ (10 µM) destabilisation in absence (control) and presence of Ser (10, 50 and

100 µM) measured by ThT assays; (B) TEM observation of pre-formed $A\beta_{1-42}$ in absence (left) or in presence (right) of 50 and 100 µM Ser, after 50 h incubation.

Figure 4. Inhibitory effects of Tryp, Trpa, Ser, Nser, Phol, 3IA, Mel and Tee (50 µM) against $A\beta_{1-42}$ cytotoxicity (5 µM) on PC12 cells, without pretreatment (NPT) and with pretreatment of 24 hours (PT). Results are expressed as mean SEM of four replicates (n = 4). * $p < 0.05$; ** $p < 0.01$ $A\beta_{1-42}$ versus compounds.

Figure 5. (A) Inhibitory effect of Ser at different concentrations on $A\beta_{1-42}$ (5 µM) induced-cytotoxicity on PC12 cells, with pretreatment of 24 hours (PT) against Aβ toxicity. Results are expressed as mean SEM of four replicates (n = 4). *$p<0.05$; ** $p<0.01$; ***$p<0.001$; **** $p<0.0001$ $A\beta_{1-42}$ versus Ser at different concentrations. (B) SIRT-1 gene expression (A.U); (C) SIRT-2 gene expression (A.U); (D) Hsp70 gene expression (A.U); (E) HO-1 gene expression (A.U) of Aβ incubated alone (5µM) or with Ser (50µM) and Ser (50µM) alone; (F) HO activity expressed by pmoles of bilirubin/mg of protein/hour of Aβ incubated alone (5µM) or with Ser (50µM) and Ser (50µM) alone. Superscript letter [a] indicates a significant difference between C vs Aβ; superscript letter [b] indicates a significant difference between C vs Ser 50 µM + Aβ: superscript letter [c] indicates a significant difference between C vs Ser 50 µM; superscript letter [d] indicates a significant difference between Aβ vs Ser 50 µM + Aβ; superscript letter [e] indicates a significant difference between Aβ vs Ser 50 µM.

Figure 1

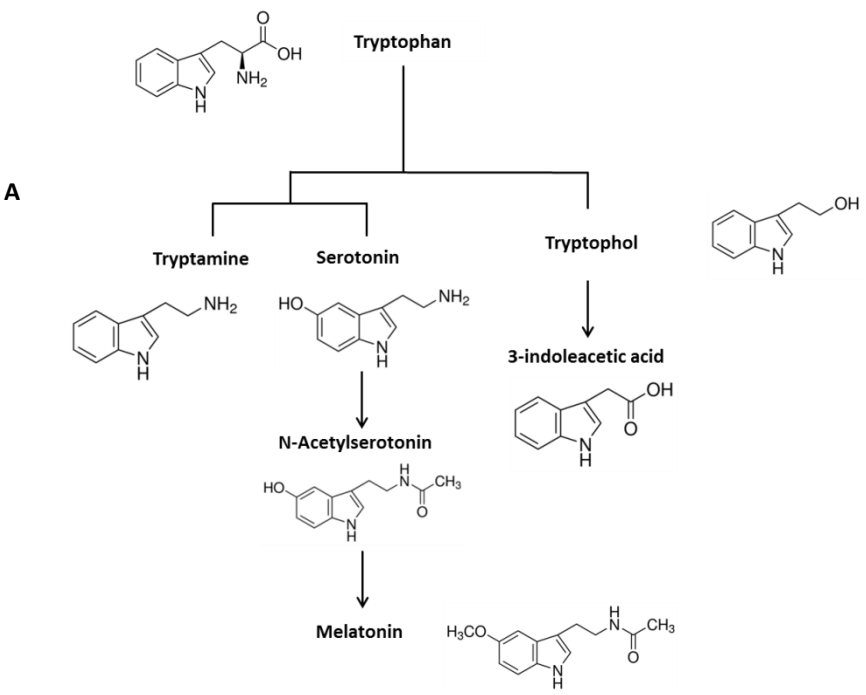
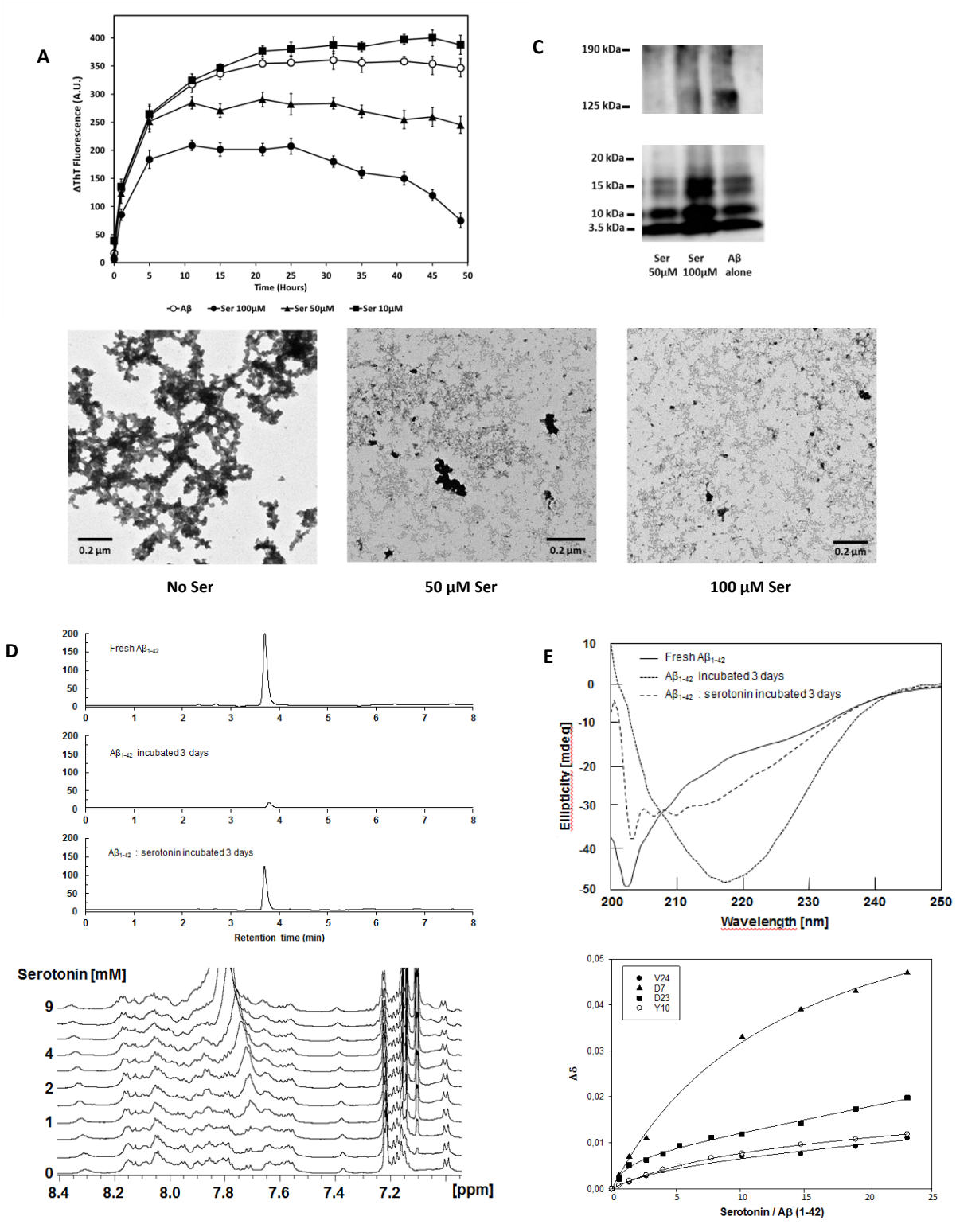
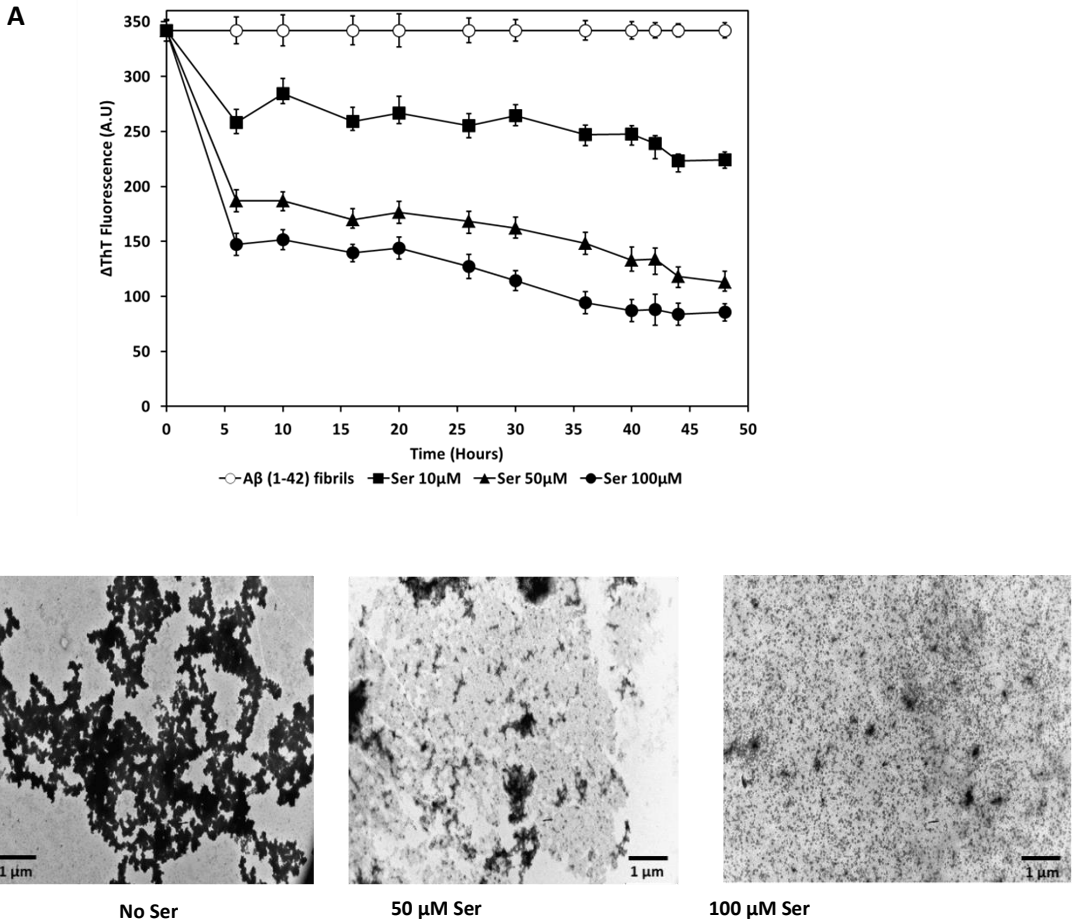

A
Tryptophan
Tryptamine
Serotonin
Tryptophol
3-indoleacetic acid
N-Acetylserotonin
Melatonin
B
ΔThT Fluorescence (A.U.)
Time (Hours)
Aβ
Tryp
Trpa
Ser
Nser
Phol

Figure 2


A
ΔThT Fluorescence (A.U.)
Time (Hours)
Aβ
Ser 100μM
Ser 50μM
Ser 10μM
C
190 kDa
125 kDa
20 kDa
15 kDa
10 kDa
3.5 kDa
Ser 50μM
Ser 100μM
Aβ alone
B
0.2 μm
No Ser
50 μM Ser
100 μM Ser
D
Fresh Aβ1-42
Aβ1-42 incubated 3 days
Aβ1-42 : serotonin incubated 3 days
Retention time (min)
E
Fresh Aβ1-42
Aβ1-42 incubated 3 days
Aβ1-42 : serotonin incubated 3 days
Ellipticity [mdeg]
Wavelength [nm]
F
Serotonin [mM]
9
4
2
1
0
8.4
8.2
8.0
7.8
7.6
7.4
7.2
[ppm]
V24
D7
D23
Y10
Δδ
Serotonin / Aβ (1-42)

765

766 **Figure 3**



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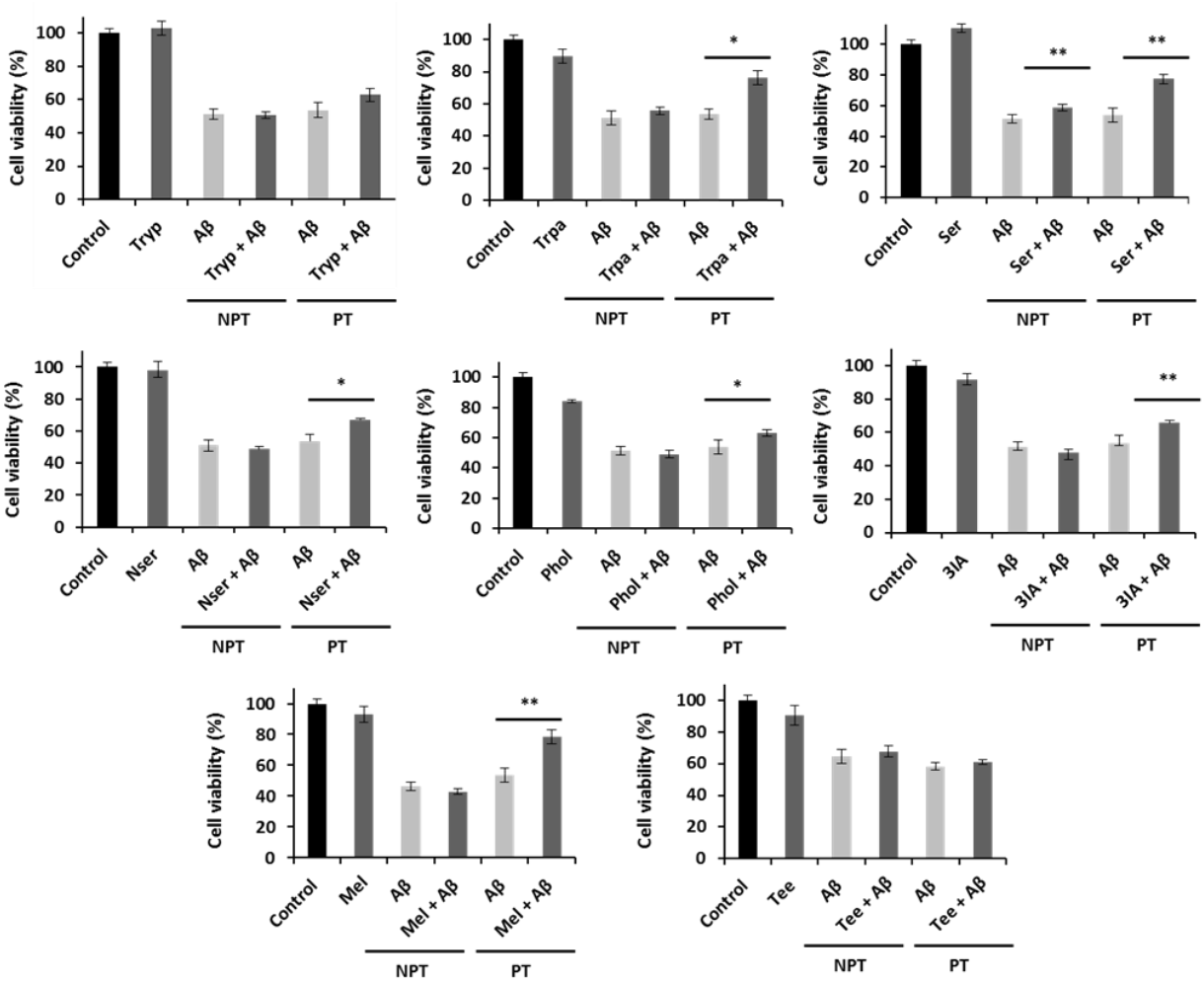
771

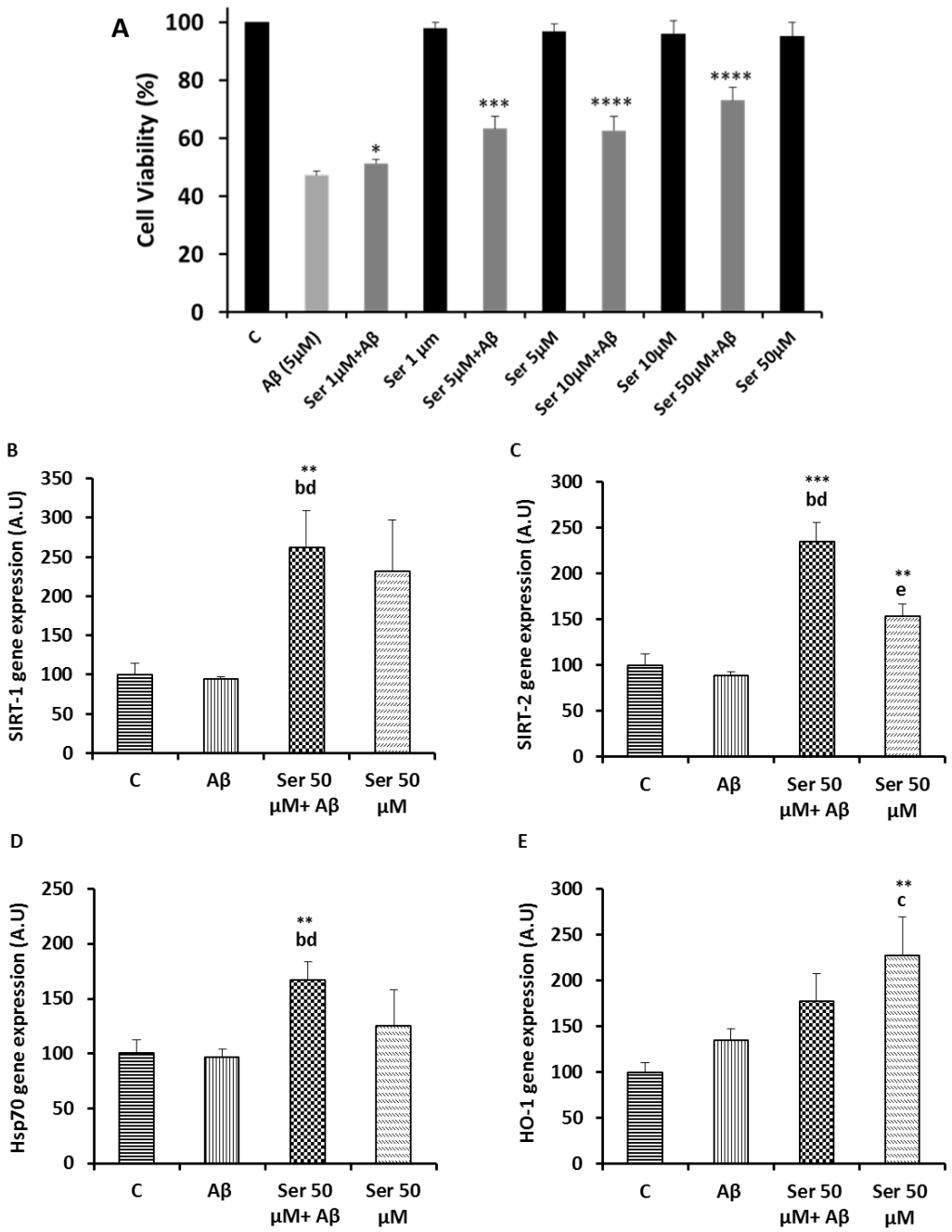
772 **Figure 4**

773



774

Figure 5


A
Cell Viability (%)
100
80
60
40
20
0
*
***
****
****
C
Aβ (5μM)
Ser 1μM+Aβ
Ser 1 μm
Ser 5μM+Aβ
Ser 5μM
Ser 10μM+Aβ
Ser 10μM
Ser 50μM+Aβ
Ser 50μM
B
SIRT-1 gene expression (A.U)
350
300
250
200
150
100
50
0
** bd
C
Aβ
Ser 50 μM+ Aβ
Ser 50 μM
C
SIRT-2 gene expression (A.U)
300
250
200
150
100
50
0
*** bd
** e
C
Aβ
Ser 50 μM+ Aβ
Ser 50 μM
D
Hsp70 gene expression (A.U)
250
200
150
100
50
0
** bd
C
Aβ
Ser 50 μM+ Aβ
Ser 50 μM
E
HO-1 gene expression (A.U)
300
250
200
150
100
50
0
** c
C
Aβ
Ser 50 μM+ Aβ
Ser 50 μM

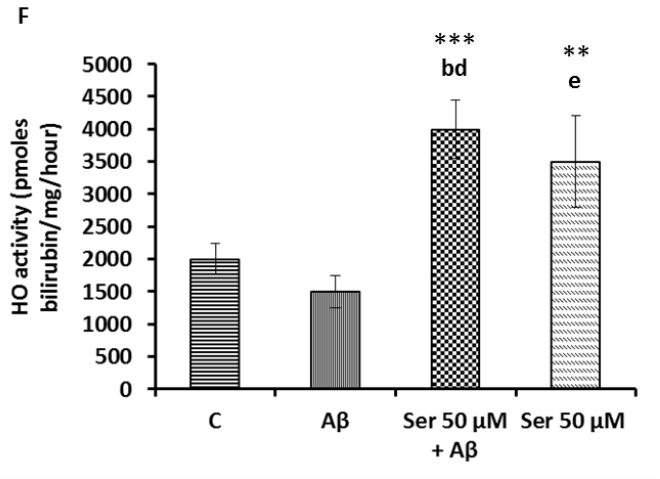


Table 1. Primers for RT-PCR.

mRNA	Primers
β-actin	Forward: 5´-TGTGATGGTGGGAATGGGTCA-3´
	Reverse :5´-TTTGATGTCACGCACGATTTCC-3´
SIRT-1	Forward: 5´TCATTCCTGTGAAAGTGATGACGA-3´
	Reverse: 5´-GCCAATCATGAGGTGTTGCTG-3´
SIRT-2	Forward: 5´-TACCCAGAGGCCATCTTTGA-3´
	Reverse: 5´-TGATGTGTGAAGGTGCCGT-3´
Hsp70	Forward: 5´- GGGCTCTGAGGAACCGAGC-3´
	Reverse: 5´-CAGCCATTGGCGTCTCTC-3´
HO-1	Forward: 5´-ACTTTCAGAAGGGTCAGGTGTCC-3´
	Reverse: 5´-TTGAGCAGGAAGGCGGTCTTAG-3´