



# Anthocyanins protect from complex I inhibition and APPswe mutation through modulation of the mitochondrial fission/fusion pathways

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## ABSTRACT

Anthocyanins are a distinguished class of flavonoids with powerful free radical-scavenging activity that have been suggested as chemotherapeutic agents for the prevention of Alzheimer disease (AD). In this study, we examined the ability of nutraceutical Medox rich in purified cyanidin 3-O-glucoside (C3G), 3-O-b-glucosides and delphinidin 3-O-glucoside (D3G) to counteract mitochondrial deficiency induced by complex I inhibition and/or amyloid- $\beta$  peptide (A $\beta$ ) induced toxicity. SH-SY5Y neuroblastoma cells were stably transfected with APP Swedish K670N/M671L double mutation (APPswe) or with the empty vector and treated with rotenone. We report that Medox treatment improves the metabolic activity and maintains cell integrity in both cell lines. At the mitochondrial level, APPswe and rotenone induced mitochondrial fragmentation, an effect that was counteracted by Medox through the modulation of fission and fusion proteins, resulting in a reshaped mitochondrial network. Although Medox was unable to fully neutralise the effects of rotenone on ATP levels and mitochondrial membrane potential, it was able to prevent rotenone-induced cytotoxicity.

Our findings suggest that Medox anthocyanins, on top of their antioxidant capacity, ameliorate mitochondrial dysfunction generated by A $\beta$  overproduction or by chemical inhibition of mitochondrial complex I via stabilization of the fusion/fission processes. Modulation of the mitochondrial network has been suggested as a novel therapeutic approach in diseases involving mitochondrial dysfunction and oxidative stress. Hence, increasing the understanding of how anthocyanins influence mitochondrial dynamics in a neurodegenerative context, could be of future therapeutic value.

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## 1. Introduction

Alzheimer disease (AD) is a devastating disorder of multifactorial etiology that leads patients into memory loss and cognitive decline. By 2035, the nearly 32 million patients suffering from this dementia worldwide is estimated to be doubled [1]. At the molecular level, AD is characterized by accumulation of amyloid- $\beta$  peptide (A $\beta$ ) in extracellular plaques and hyperphosphorylated tau protein in intra-neuronal tangles in brain. Today, no treatment can reverse the progression of AD, and available drugs can only ameliorate some symptoms once the pathology is diagnosed [2,3]. The scientific community is exerting a tremendous effort on conducting new disease-modifying strategies to delay its onset and maintain the independency of the diagnosed patients.

Anthocyanins are flavonoids belonging to a large natural group of hydrophilic pigments, known as polyphenols, that are ubiquitously found in the plant kingdom [4]. Recent epidemiological and pre-clinical research have suggested the health benefits of dietary anthocyanin-rich extracts in reducing incidence of AD [5,6,7,8]. It has been suggested that, by crossing the brain blood barrier (BBB) [9], anthocyanins may accomplish their ROS scavenging ability and modulate signaling pathways in the central nervous system [10,11]. Furthermore, the presence of intact glycosylated anthocyanins such as cyanidin-3-O-beta-glucoside (C3G) and delphinidin-3-O-beta-galactoside (D3G) in the hippocampus of adult rats leads to enhanced cognitive performance [12] and improved spatial memory as well as reduced lipid peroxidation, higher levels of reduced glutathione, and the induction of antioxidant enzymes [13].

Medox capsules, a nutraceutical compound that consists of natural anthocyanins purified from bilberry (*Vaccinium myrtillus*) and black currant (*Ribes nigrum*) have been examined in clinical studies where it has diverse health-promoting effects by modulating pro-inflammatory chemokines [14] and HDL-cholesterol/LDL-cholesterol concentrations in plasma [15]. The characterization of the different anthocyanins in cellular models of Parkinson's disease (PD) and AD, revealed a differential

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impact on protective responses due to a biochemical structure-activity relationship [16]. Synergistic interactions involving multiple anthocyanins have been proposed for maximal neuroprotection [17]. In PD cellular models, D3G (but not C3G) was shown to protect primary neurons from the toxic effects of rotenone [16] whereas in SH-SY5Y cellular models of AD, C3G was reported to inhibit  $A\beta_{25-35}$ -induced intracellular ROS formation [18] as well as the toxicity of oligomeric and fibrillar  $A\beta_{1-42}$  [19].

Mitochondrial deficiency is a prevailing feature of AD, wherein  $A\beta$  contributes by significantly reducing brain metabolism, increasing oxidative stress [20] and an imbalanced mitochondrial fission and fusion that results in abnormal mitodynamics and increased sensitivity to cell death [21]. In addition to the above mentioned study [16], recent evidence suggests that flavonoids can prevent mitochondrial dysfunction-associated pathologies by inhibiting  $H_2O_2$  overproduction in damaged brains [22].

In the present study, we investigated whether Medox anthocyanins could mitigate the toxicity of mitochondrial complex I inhibition by rotenone in a cellular AD model. Rotenone has been shown to reduce cell viability and mitochondrial membrane potential ( $\Delta\psi_m$ ), and induce mitochondrial fragmentation and autophagy in previous studies [23,24]. To explore Medox effects, we performed experiments using SH-SY5Y cells stably transfected with the familial AD APP Swedish KM670/671NL double mutation (APP<sup>swe</sup>). Our results demonstrate that the protective mechanisms of Medox involve an improvement of  $\Delta\psi_m$ , cell membrane integrity and mitochondrial dynamics.

## 2. Material and methods

### 2.1. Reagents

Dulbecco's Modified Eagle Medium (DMEM; 11966025), fetal bovine serum (FBS), TrypLE™ Express, Geneticin® Selective Antibiotic (G418 Sulfate), and bicinchoninic protein assay (Pierce™ BCA) kit were obtained from Life Technologies, (CA, USA). Resazurin sodium salts suitable for cell culture, Rotenone  $\geq 95\%$  and dimethyl sulfoxide for molecular biology and D-(+)-Galactose ( $\geq 99\%$ ) were obtained from Sigma-Aldrich (MO, USA). The mitochondrial ToxGlo™ assay kit was purchased from Promega Corporation (Madison, WI). Complex IV Human Specific Activity Microplate Assay Kit was obtained from Abcam (Cambridge, UK).

### 2.2. Primary antibodies

The following antibodies were used in this study: mouse anti-Opa1 (1:1000; BD Biosciences, San Jose, CA); mouse anti-Mfn2 (1:1000; Abcam, Cambridge, UK); mouse anti-Drp1 (1:500; BD Biosciences, San Jose, CA); rabbit anti- $\beta$ -actin (1:5000; Sigma, MO, USA).

### 2.3. Medox anthocyanins

The nutraceutical, Medox®, has been developed by Biolink Group (Sandnes, Norway). Medox capsules consist of natural purified anthocyanins isolated from bilberry (*Vaccinium myrtillus*) and black currant (*Ribes nigrum*). The concentration assessed for the study is expressed in  $\mu\text{g/ml}$ . The relative content of each anthocyanin in the capsules was 33.0% of 3-O- $\beta$ -glucosides, 3-O- $\beta$ -galactosides, and 3-O- $\beta$ -arabinosides of cyanidin; 58.0% of 3-O- $\beta$ -glucosides, 3-O- $\beta$ -galactosides, and 3-O- $\beta$ -arabinosides of delphinidin; 2.5% of 3-O- $\beta$ -glucosides, 3-O- $\beta$ -galactosides, and 3-O- $\beta$ -arabinosides of petunidin; 2.5% of 3-O- $\beta$ -glucosides, 3-O- $\beta$ -galactosides, and 3-O- $\beta$ -arabinosides of peonidin; 3.0% of 3-O- $\beta$ -glucosides, 3-O- $\beta$ -galactosides, and 3-O- $\beta$ -arabinosides of malvidin; and 1.0% of 3-O-rutinoside of cyanidin and delphinidin. The capsules also contained pullulan, maltodextrin, and citric acid (4%) to maintain the stability of anthocyanins.

### 2.4. Cellular culture and treatments

Human SH-SY5Y cells were obtained from American Type Culture Collection (ATCC, USA). Stable transfection of APP with the Swedish KM670/671NL double mutation or empty pcDNA 3.1 vector used as control were performed as formerly described [25]. SH-SY5Y transfected cells were routinely grown at 37 °C in a humidified incubator with 5%  $CO_2$  in DMEM + Glucose (10 mM) or + Galactose (10 mM), supplemented with 10% fetal bovine serum and geneticin. All experiments were performed after 24 h of incubation. Cells were treated with Medox anthocyanins (0.05  $\mu\text{g/ml}$ ; containing 20 nM C3G and 30 nM D3G) for 18 h. To study Medox effect against rotenone-induced toxicity, cells were incubated for 18 h with rotenone (100 nM) in presence or absence of Medox anthocyanins. To explore whether Medox was able to improve cell recovery after a short exposure to rotenone, cells were exposed to rotenone (100 nM) for 30 min and then incubated in the presence or absence of Medox anthocyanins for 18 h.

### 2.5. Resazurin assay

The Resazurin reagent was prepared in DMEM + glucose (10 mM) or galactose (10 mM) at a final concentration of 20  $\mu\text{g/ml}$ . SH-SY5Y transfected cells were seeded in 24-well plates at  $10 \times 10^4$  cells/well. After experimental treatments in glucose or galactose containing media, plates were washed once in  $1 \times$  PBS and incubated in 400  $\mu\text{l}$  of Resazurin working solution for 2 h at 37 °C. Fluorometric Resazurin reduction was measured in Tecan plate readers and the values were calculated by Magellan™ Data Analysis Software. Results were expressed as percentages of the values obtained from the appropriate controls.

### 2.6. Measurement of complex IV activity

The complex IV specific activity microplate assay kit (ab 1099910) was used to determine the activity and quantity of the enzyme in SH-SY5Y transfected cells. Cells were seeded in 6-well plates at  $15 \times 10^4$  cells/well in glucose-containing media and treated in the absence or presence of Medox (0.05  $\mu\text{g/ml}$ ) for 18 h as previously described. The assay was performed following the manufacturer's instructions. Complex IV activity and protein quantity were determined colorimetrically at 550 nm and 405 nm respectively. The activity values were calculated by Magellan™ Data Analysis Software. Results were expressed by OD/min in relation to protein levels.

### 2.7. Measurement of cell membrane permeability and ATP levels

The mitochondrial ToxGlo™ assay kit (G80000) was used to assess mitochondrial deficits. Two different passages of control and APP<sup>swe</sup> transfected cells were seeded by duplicate in three independent 96-well plates at  $5 \times 10^4$  cells/well in galactose-containing media. Cell membrane permeability was first assessed by the presence of bis-AAF-R110, a fluorogenic peptide substrate associated with protease activity, that gives significant signal with non-viable cells, in relation to viable cells. Results are expressed as fluorescence intensity. Next, ATP was measured over the same cell cultures by adding the ATP Detection Reagent, resulting in cell lysis and generation of a luminescent signal proportional to the amount of ATP present. The concentration of ATP was calculated from a calibration curve described in the kit and the data was expressed by nmoles of ATP.

### 2.8. Analysis of mitochondrial membrane potential ( $\Delta\psi_m$ ) by flow cytometry

Cells were seeded in 12-well plates at  $10 \times 10^4$  cells/well in galactose containing medium and treated with rotenone and Medox as previously described. After treatment, cells were washed with PBS and stained with 100 nM tetramethyl rhodamine methyl ester (TMRM, Life Science Technologies) for 15 min at 37 °C, then washed with PBS. The cells were

detached by TrypLE (Thermo Fisher Scientific), collected in 500  $\mu$ l PBS supplemented with 0.1% Bovine Serum Albumine (Sigma Aldrich), and transferred to FACS tubes and kept at 4 °C until measurement. Cytofluorimetric analysis was performed using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA) equipped with a 488 nm argon laser. TMRM signal was analyzed in the FL2 channel and the data were acquired on a logarithmic scale. 10,000 events from live cells were collected for each analysis. Data were analyzed using the CellQuest software (BD) and exposure to 10  $\mu$ M CCCP (3-chlorophenylhydrazon) for 10 min was used to set a threshold of fluorescence intensity for those cells with intact  $\Delta\psi_m$ . Results are expressed as % of all cells with TMRM fluorescence greater than the threshold set by CCCP.

## 2.9. Analysis of apoptosis by flow cytometry

Apoptotic cell death was quantified with a FITC-conjugated annexin V and propidium iodide (PI) kit (Thermo-Fisher, Massachusetts, USA). Transfected SH-SY5Y cells were seeded in 12-well plates at  $10 \times 10^4$  cells/well. After the treatments, cells were detached with TrypLE Express Enzyme (Thermo-Fisher, Massachusetts, USA), centrifuged (500  $\times$  g, 5 min) and re-suspended in PBS. They were then stained according to the manufacturer's instructions. FITC and PI fluorescence was measured on a BD Accuri C6 plus flow cytometer (Becton Dickinson Biosciences, New Jersey, USA) equipped with a 488 nm laser (FL1 533/30 and FL2 585/40 filters respectively). Annexin V–FITC positive cells that were PI negative were considered to be apoptotic. The results were expressed as % of apoptotic cells out of a total of 50,000 events per condition.

## 2.10. Western blot for mitochondrial dynamic status

Transfected SH-SY5Y cells were seeded in 6-well plates at  $15 \times 10^4$  cells/well in glucose-containing medium. After treatments, cells were washed twice in  $1 \times$  PBS and lysed in a cell lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA) containing anti-protease and anti-phosphatase cocktail (Sigma Aldrich, Stockholm, Sweden). Then they were sonicated and centrifuged for 10 min at 12,000 rpm to remove cell debris. 50  $\mu$ g of protein fraction was mixed with tricine loading buffer (0.16 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.01% bromophenol blue, 0.1 M DTT added fresh), heated and separated by SDS-PAGE using 10% acrylamide gels. Proteins were then transferred to a nitrocellulose membrane (0.2  $\mu$ m pore size membrane Protran® BA83, Whatman, (GE Healthcare, Uppsala, Sweden) and blocked for 1 h in Tris-buffered solution containing 0.1% Tween-20 and 5% (w/v) dried milk (BCR685 FLUKA) (Sigma Aldrich, Stockholm, Sweden). The membranes were incubated with the primary antibody described above in Tris-buffered solution containing 0.1% Tween-20 followed by anti-rabbit or anti-mouse immunoglobulin G (IgG) horseradish peroxidase (1:5000; GE Health Care, Little Chalfont, UK). Immunoreactivity was detected by the ECL detection system (Amersham Biosciences, Little Chalfont, UK). The optical density of immunoreactive bands was calculated by ImageJ 1.383 software (NIH, MA, USA). Each experiment was performed 4 times. Results were expressed as percentages of the values obtained from the appropriate controls and normalized by  $\beta$ -actin expression.

## 2.11. Confocal microscopy

Cells were seeded on cover slips coated with poly-D-lysine. After treatments in glucose-containing media, mitochondria were stained with 200 nM Mitotracker CMXRos (Molecular Probes Inc.) dissolved in media for 30 min at 37 °C and 5% CO<sub>2</sub> followed by incubation for 30 min in fresh media supplemented with 10% FBS. After a brief washing in PBS, cells were fixed with 4% paraformaldehyde, washed and mounted with ProLong Gold Antifade reagent (Molecular Probes, Inc.) Cells were examined with the 40 $\times$  objective using an inverted Laser Scanning Microscope (LSM 510 META, Zeiss, Germany).

## 2.12. Statistical analysis

Statistical analyses of means were performed using one-way analysis of variance (ANOVA) followed by Tukey or Bonferroni post hoc tests, as specified in the legend of each figure. The null hypothesis was rejected at  $P$ -value < 0.05. All statistical computations were carried out using GraphPad Prism Software, Version 5.0 (<http://www.graphpad.com/prism/Prism.htm>). Data values are expressed as mean  $\pm$  standard error mean (SEM).

## 3. Results

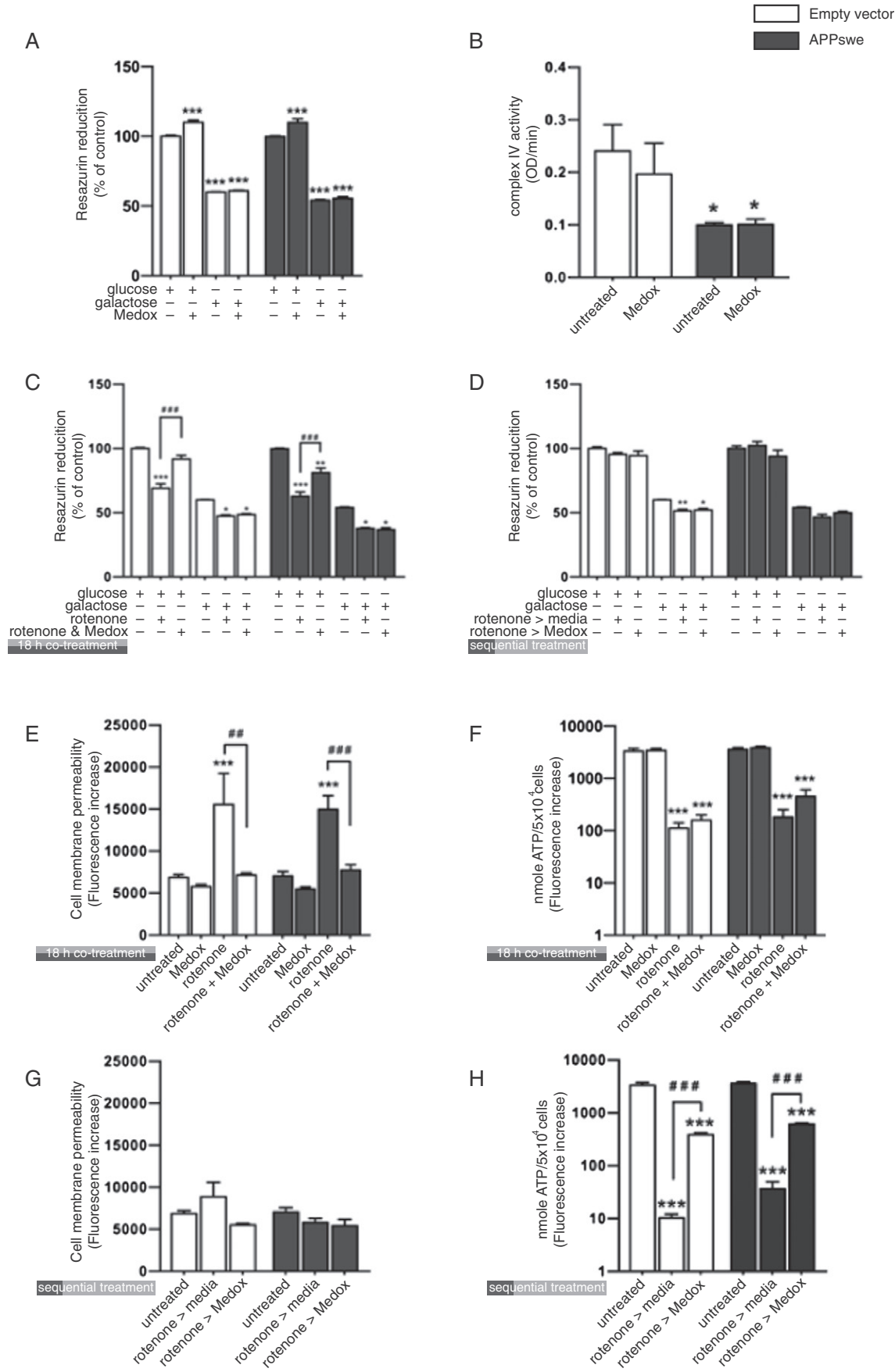
### 3.1. Medox anthocyanins counteract the rotenone induced impairment of mitochondrial function

We first aimed to determine the effect of Medox on cellular NADH levels by using the Resazurin reduction assay as an indicator of cellular metabolic activity. We found that, under conditions of normal glucose concentrations, 0.05  $\mu$ g/ml of Medox anthocyanins increased NADH levels both in control and APPswe cells compared with their respective untreated conditions (Fig. 1A, see Medox dose-response curves in Fig. S1). Medox treatment (0.05  $\mu$ g/ml, 24 h) was not toxic to the cells, as determined by the annexin/PI assay (Fig. S2). To dissect the effects of Medox on mitochondrial function, we also performed experiments in galactose containing media. This discards the contribution of glycolytically generated NADH [26] and thus Resazurin reduction is a more direct indicator of mitochondrial activity. As expected, we found a significant decrease in cell metabolism when galactose was used instead of glucose (Fig. 1A). However, under these conditions, Medox was not able to improve the metabolic activity of the cells (Fig. 1A).

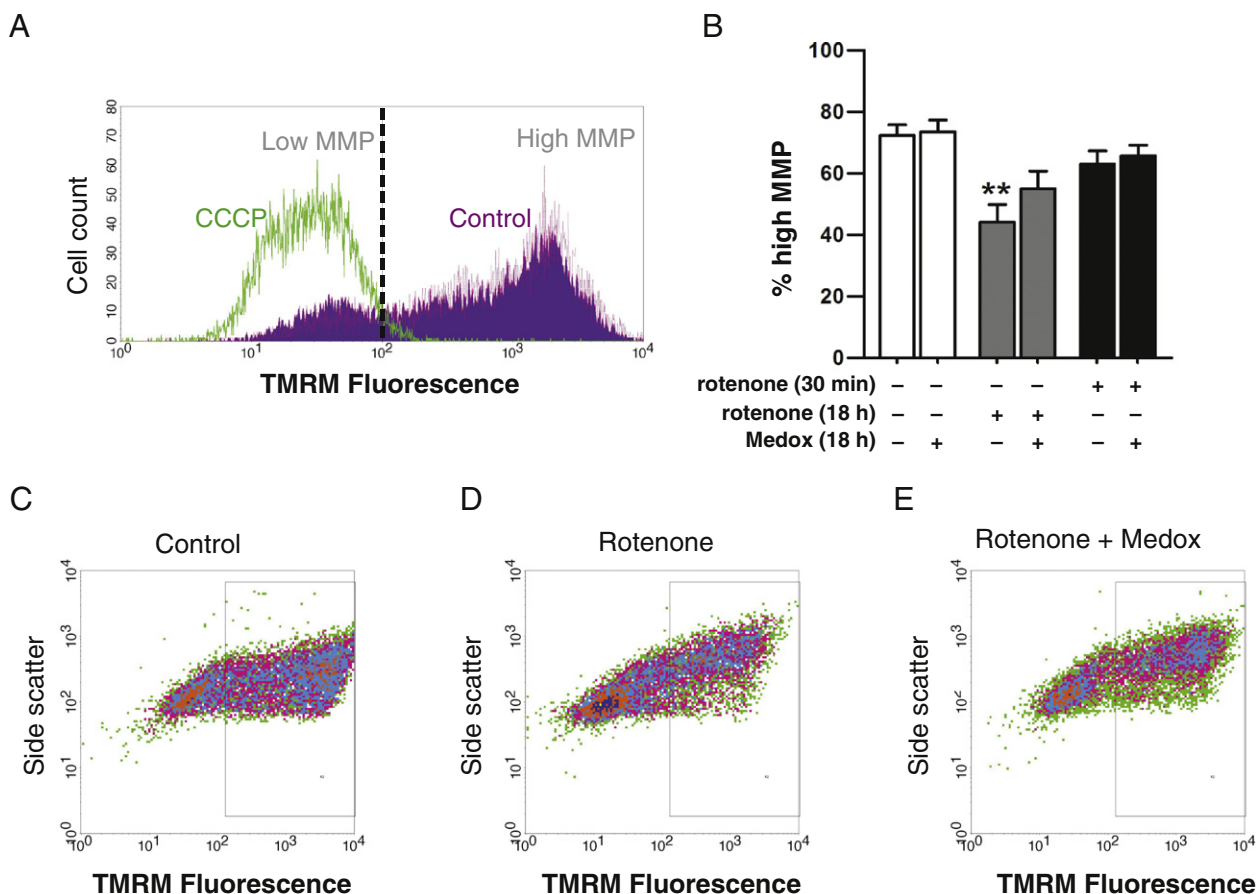
As seen in Fig. 1B, APPswe cells showed a significant lower activity of complex IV compared to controls. Treatment with Medox (0.05  $\mu$ g/ml, 24 h) did not modify basal complex IV activity in any of the cell types.

Fig. 1C shows the effects of the complex I inhibitor rotenone (100 nM, 18 h) on Resazurin reduction in controls and APPswe cells. In the presence of glucose, rotenone significantly decreased the metabolic activity in both cell types (Fig. 1C). This effect was fully neutralized by co-treating with Medox in controls and partially in APPswe cells. On the contrary, in galactose containing media, Medox was unable to counteract the rotenone effects in both cell types. To explore whether Medox could improve cell recovery after a short blockage of complex I, we first treated cells with rotenone (100 nM) for 30 min, followed by 18 h Medox treatment. In contrast to the results seen in Fig. 1C, a 30 min treatment with rotenone did not induced major changes in Resazurin

**Fig. 1. Medox counteracts the rotenone induced impairment of mitochondrial function.** (A) SH-SY5Y cells stably transfected with APPswe or empty vector (control cells) treated with Medox (18 h) increase cellular NADH levels (measured by Resazurin reduction) when cultured in glucose containing media but not when glucose was replaced by galactose. (B) Compared to controls, APPswe cells showed significant reduction in complex IV activity that was not restored by Medox (18 h). (C) Co-treatment with Medox (18 h) inhibits the rotenone-induced decrease in NADH levels in glucose containing media but not in galactose containing media. (D) Short exposure to rotenone (30 min) followed by incubation with fresh media for 18 h produced major changes cellular NADH levels. (E–H) Co-treatment (18 h) with Medox normalized rotenone induced permeability of the cell membrane (E) but failed to normalize the effects on ATP depletion (F). (G) Short exposure to rotenone followed by incubation with fresh media  $\pm$  Medox (18 h) did not have any effect on cell membrane permeability. (H) Reduced ATP production by short exposure to rotenone (30 min) was partially restored by Medox (18 h) post-treatment in both control and APPswe cells. White bars correspond to control (empty vector) SH-SY5Y cells and black bars correspond to APPswe transfected cells. Medox was used at a concentration of 0.05  $\mu$ g/ml. Rotenone was used at 100 nM. Data are presented as mean  $\pm$  SEM;  $n = 4$ –6 independent experiments; \* $p < 0.05$  \*\* $p < 0.01$  \*\*\* $p < 0.001$  vs untreated cells and ## $p < 0.01$  and ### $p < 0.001$  vs rotenone values at ANOVA with Bonferroni post hoc test.







**Fig. 2. Medox partly restores mitochondrial membrane potential.** Mitochondrial membrane potential was measured using FACS in live vector transfected cells stained with TMRM. The results are expressed as mean number of cells with TMRM fluorescence above threshold set by depolarizing one set of cells with CCCP (10 mM, 10 min; see shift in Fig. A). (B) Cells were exposed to 18 h rotenone (100 nM)  $\pm$  Medox (0.05  $\mu$ g/ml) or rotenone (100 nM, 30 min) followed by  $\pm$  Medox (18 h) in galactose-supplemented media. Rotenone decreased  $\Delta\psi_m$  and this effect was reversed by Medox. The data are presented as mean  $\pm$  SEM;  $n = 5$  independent experiments;  $**p < 0.01$  vs untreated at ANOVA with Tukey post hoc test. (C–E) Representative flow cytometry dot plots showing TMRM fluorescence in SH-SY5Y cells incubated with control condition (C), rotenone (D) and rotenone + Medox (E).

reduction. Only a minor effect was found in control cells when the experiments were done in galactose containing media (Fig. 1D). The addition of Medox for 18 h did not change these effects (Fig. 1D).

We next measured cell membrane permeability and ATP levels using both experimental set-ups described above; long co-treatment with Medox and rotenone, or short exposure of rotenone followed by a wash-out period with media or Medox. Medox itself, did not alter ATP production or membrane permeability. On the contrary, rotenone (100 nM, 18 h) increased cell permeability and reduced ATP production in both cell types (Fig. 1E and F). Co-treatment with Medox was able to restore membrane permeability (Fig. 1E), but not ATP production (Fig. 1F).

In the short exposure to rotenone followed by a 18 h wash-out period with media we detected no major effects on cell membrane permeability (Fig. 1G), but a drastic inhibition of ATP levels. This effect was counteracted with the addition of Medox (Fig. 1H).

Since mitochondrial function is highly dependent on the mitochondrial membrane potential, we further analyzed the effects of Medox on  $\Delta\psi_m$  by flow cytometry. The cationic, fluorescent dye TMRM was utilized to quantify the  $\Delta\psi_m$  based on its specific mitochondrial accumulation in polarized mitochondria. Fig. 2A shows our settings to discriminate between low (CCCP depolarized mitochondria) and high  $\Delta\psi_m$  (untreated cells). As shown in Fig. 2B, 18 h rotenone treatment significantly decreased the number of mitochondria with high membrane potential, an effect that was not seen at the 30 min treatment. Co-treatment with Medox slightly inhibited the 18 h rotenone effects on  $\Delta\psi_m$  (Fig. 2B). Lower panel shows representative density plots from

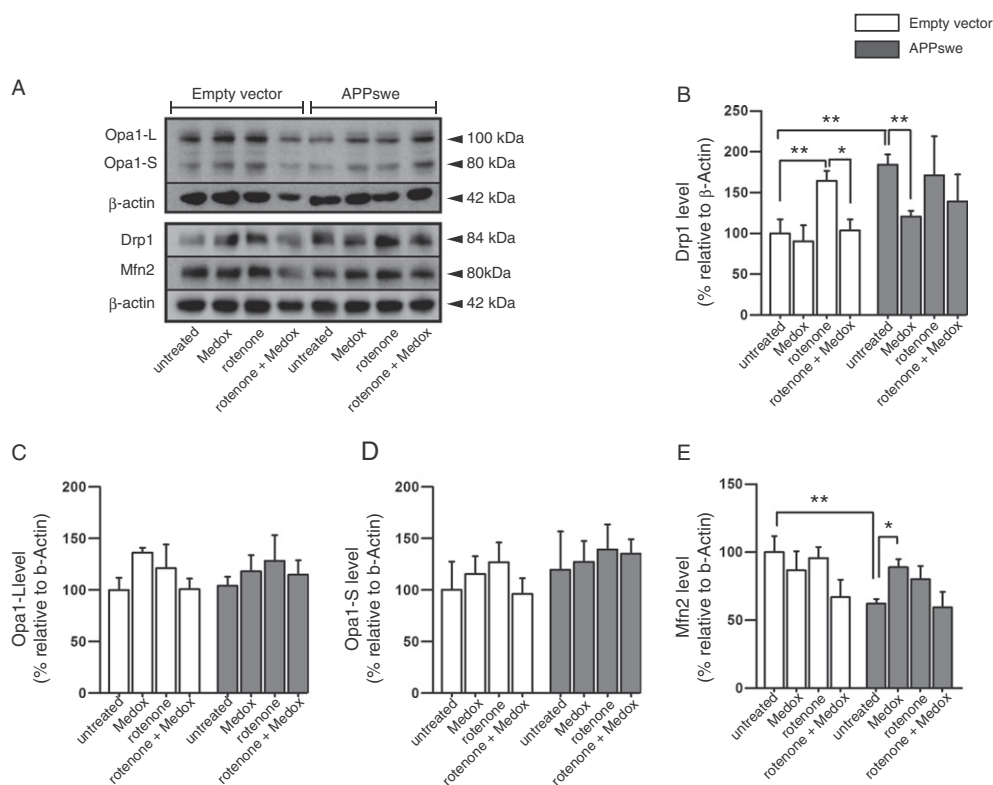
untreated (Fig. 2C), rotenone treated (Fig. 2D), and rotenone + Medox treated (Fig. 2E) cells.

### 3.2. Medox anthocyanins modulate mitochondrial dynamic proteins and counteract rotenone induced fragmentation

The responses to oxidative stress and metabolic needs are largely mediated by mitochondrial fission and fusion processes in the cells. We next examined how rotenone (18 h, 100 nM) and Medox anthocyanins (18 h) affect the expression of the mitochondrial fission protein dynamin-related protein 1, (Drp1), as well as the fusion proteins optic atrophy 1, (Opa1) and mitofusin 2, (Mfn2) (Fig. 3). In control cells, rotenone induced a significant ( $p < 0.05$ ) increase in Drp1 that was counteracted by Medox co-treatment (Fig. 3A and B). APPsw cells showed higher basal levels of Drp1 compared to controls that were not further increased by rotenone. However, Medox significantly lowered the high Drp1 levels seen in APPsw cells (Fig. 3A and B).

Opa1 is a heterotrimeric complex located in the inner membrane that contains an integral long form (~100 kD; Opa1-L) and short form (~80 kD; Opa1-S) both required for mitochondrial fusion. Rotenone or Medox treatments did not affect the expression levels of fusion proteins Opa1-L, Opa1-S or Mfn2 (Fig. 3A, C, D and E). Mfn2 levels were lower in APPsw cells compared to controls ( $p < 0.05$ ), an effect that was counteracted by Medox (Fig. 3A and E).

As shown in the micrographs and the quantification of morphological features (Fig. 4), control cells had an interconnected, normal mitochondrial network as visualized by staining with mitotracker



**Fig. 3. Medox modulates the effects induced by rotenone or APPsw mutation on mitochondrial dynamic proteins.** (A) Representative western blot images of fission (Drp1) and fusion (Opa1-L, Opa1-S and Mfn2) and  $\beta$ -actin proteins. (B–E) Histograms showing the quantification of the immunoreactivity measurements with data normalized against loading control ( $\beta$ -actin) and expressed as means  $\pm$  SEM and as a percentage of untreated cells.  $N = 3$ –4 independent experiments; \*\* $p < 0.01$ , \* $p < 0.05$ . ANOVA with Tukey post hoc test.

(Fig. 4B), whereas the APPsw transfected cells had a more irregular network at basal conditions with multiple fragmented mitochondria (Fig. 4F). Rotenone treatment increased the number of fragmented mitochondria in both controls and APPsw cells (Fig. 4A, D and H). Co-treatment with Medox was able to reduce mitochondrial fragmentation and partially restore the network in APPsw cells (Fig. 4I), an effect that was statistically significant when quantifying the amount of cells with fragmented mitochondria (Fig. 4A). The less dramatic rotenone effects in control cells were however not reduced by Medox (Fig. 4A and E).

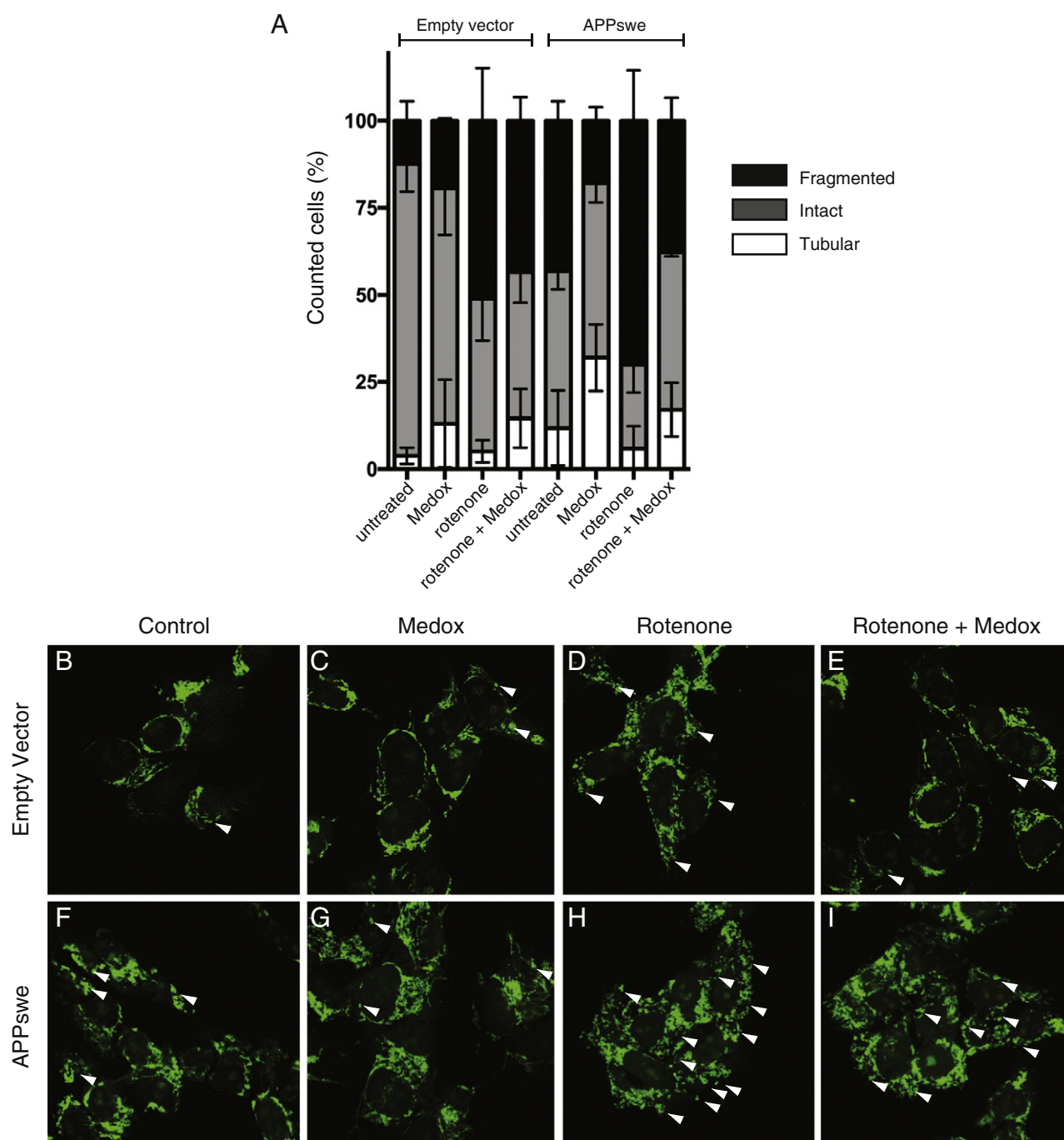
#### 4. Discussion

Anthocyanins are potent antioxidants that are implicated in a number of biological pathways linked to decreasing the risk of chronic diseases such as certain cancers, coronary disease, metabolic syndrome, type II diabetes, neurodegenerative diseases, and inflammation [8]. Several intracellular mechanisms including mitogen-activated protein kinase, nuclear factor  $\kappa$ B, AMP-activated protein kinase and Wnt/ $\beta$ -catenin are believed to mediate the protective effects of anthocyanins [8]. In this study, we have explored the possibility that Medox anthocyanins could enhance mitochondrial function in response to complex I inhibition or overproduction of A $\beta$  in an AD *in vitro* model. Numerous *in vitro* and *in vivo* studies previously stated that bioenergetic impairment of mitochondria plays a critical role in the progression of AD pathogenesis [27]. Mitochondria actively divide and fuse in response to changes in energy demands and oxidative stress [28] and accumulating evidence shows that mitochondrial dynamics is of importance for neurodegenerative processes [29,30].

We report that Medox is able to reduce rotenone- and APPsw-induced mitochondrial fragmentation, restoring the mitochondrial network. The presence of APPsw resulted in the upregulation of Drp1 and downregulation of Mfn2, two features that are associated with mitochondrial fragmentation [31–33]. Increased Drp1 levels were also

observed in control cells treated with rotenone, which is in line with the mode of action of this mitochondrial toxin. In agreement with our results, a previous study showed that N2a cells overexpressing the APPsw mutation had decreased levels of both Mfn1 and Mfn2, accompanied by mitochondrial fragmentation [34]. Similarly, rotenone was previously reported to increase Drp1 fission protein in primary neurons [35]. We show that Medox was able to normalize the effects from APPsw and rotenone on both Drp1 and Mfn2. The proteolysis of Opa1 to smaller isoforms lead to the inactivation of mitochondrial fusion [36]. Kelsey et al. [37] reported that anthocyanins attenuated Opa1 cleavage and thereby reduce mitochondrial fragmentation in cerebellar granule neurons [37]. In our study, we found that neither rotenone, the presence of APPsw or Medox altered Opa1 levels or proteolysis.

Fused mitochondrial network is associated with respiratory active cells, while mitochondrial fragmentation results in mitochondrial dysfunction in mammalian cells [38]. In AD patients, the bioenergetic deficits are associated to a decreased activity of mitochondrial enzymes such as cytochrome c oxidase (mitochondrial complex IV) [39], pyruvate dehydrogenase [40] and  $\alpha$ -ketoglutarate dehydrogenase [41]. We found a significant reduction in mitochondrial complex IV activity in APPsw cells, that was not recovered by Medox anthocyanins. Similarly, Lagoa et al. [22] reported that other flavonoids (epicatechin, quercetin and kaempferol) did not modify the redox state of complex IV, but reduced cytochrome c. As anthocyanins have a chemical homology with quinone moiety CoQ, they were found to increase complex I activity [42]. We used a paradigm of rotenone-induced mitochondrial impairment [43] in glucose or galactose conditions to explore whether the effects of Medox are dependent on mitochondrial function. The presence of glucose drives the cells to generate ATP mainly by glycolysis and TCA cycle. By replacing glucose with galactose, the contribution of glycolysis is reduced and cells are more dependent on mitochondrial energy production through the respiratory chain. In galactose containing media,



**Fig. 4. Medox normalizes the mitochondrial network alterations resulting from rotenone and APPswe mutation.** Mitochondria were stained with a fluorescent marker (Mitotracker) to explore mitochondrial morphology in the presence of Medox (0.05  $\mu\text{g}/\text{ml}$ ) or rotenone (100 nM) alone or in co-treatment for 18 h in control and APPswe cells. **(A)** Quantification of immunofluorescent stained cells assigned to one of three morphological categories; intact, tubular or fragmented. Blinded counts of 75–300 cells from three independent experiments were performed in cell lines overexpressing APPswe or empty vector. The categories are expressed as a percentage of the total counted. Data are mean  $\pm$  SEM. Co-treatment with rotenone and Medox significantly decreased the percentage of cells with fragmented mitochondria compared to rotenone treatment alone in APPswe cells (\* $p < 0.05$ , one-way ANOVA and Tukey–Kramer multiple comparisons test). **(B–I)** Representative images of mitochondrial network at different conditions in both cell types. Arrowheads point to fragmented mitochondria in the micrographs.

Medox was unable to protect against the effects of a long term rotenone treatment (18 h, 100 nM) on metabolic activity. This lack of effect was seen in both control and APPswe cells. However, in glucose-containing media Medox fully counteracted the rotenone effects on metabolic activity in control cells, suggesting that exogenous anthocyanins can replace the antioxidant activity of NADH [43], leading to an increase in reduced NADH levels. Nevertheless, in APPswe cells, this Medox-mediated protection was limited, possibly due to their intrinsically higher levels of oxidative stress triggered by the accumulation of A $\beta$

[25]. Similarly, C3G was reported to only partially protect from the pro-necrotic and apoptotic effects of A $\beta_{25-35}$  in SH-SY5Y cells [18].

In galactose-containing media, Medox anthocyanins prevented the effects of long-term rotenone treatment in cell membrane permeability, both in controls and APPswe cells. Interestingly, Medox improved  $\Delta\psi_m$ , but not ATP levels. As expected, a short (30 min) treatment with rotenone followed by a wash-out period of 18 h had milder effects on cell viability, as seen by the lack of effects on cell membrane permeability and metabolic activity. However, compared to an 18 h constant

rotenone treatment, the transient 30 min rotenone treatment resulted in stronger ATP depletion that was partially counteracted by Medox. This intriguing result may suggest that in the shorter treatment ATP consumption might take place to compensate the survival needs. Thus, in the beginning of the rotenone treatment, cells could transiently maintain the mitochondrial membrane potential by reversing the ATP synthase [44]. In these conditions, when the metabolic activity is diverted towards anaerobic glycolysis for ATP production [45], the antioxidant role of Medox might be essential for preventing the associated increase in oxidative stress and thus for cell survival. Indeed, it has been reported that oxidative stress is more relevant than ATP depletion for apoptotic cell death mediated by the inhibition of the respiratory chain [46].

Regarding the effects on ATP, an interesting finding was the ability of Medox anthocyanins to recover mitochondrial ATP depletion elicited by short exposure (30 min) but not by longer (18 h) exposure to rotenone. Our data are consistent with a previous study from Skemienė et al. [42] which showed that the two main anthocyanins in Medox (C3G and D3G) increased the activity of complex I in ischemia-damaged mitochondria, resulting in enhanced ATP synthesis. C3G and D3G were reported to interact with complex I and, together with CoQ, participate as electron acceptors stimulating respiration and ATP production [42]. On the other hand, rotenone binds with complex I in a site located above the anthocyanins-binding site [40]. Thus, the possibility that Medox and rotenone could compete sterically for the same binding site in complex I could be suggested. Indeed, our data show a several fold increase in ATP levels when Medox is applied after short rotenone exposure, but not when it is applied in combination with rotenone.

The maintenance of energy state (ATP/ADP ratio) and healthy mitochondrial network are largely dependent on coordinated adaptive mechanisms that involve mitogenesis/mitophagy and fission/fusion pathways [28]. Due to the cellular benefits in maintaining a functional mitochondrial network, therapeutic approaches to target proteins involved in modulating mitochondrial dynamics have been suggested as key features for several disorders with oxidative stress and mitochondrial dysfunction [47,48]. Anthocyanins have been shown to induce autolysosome formation, targeting mitochondria to mitophagy pathways under chronic toxicity conditions [49]. Moreover, a recent study in hepatic mitochondria demonstrated that anthocyanins improved mitochondrial defects by inducing mitochondrial biogenesis through the activation of AMP-activated protein kinase (AMPK)/peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) pathway [50]. Furthermore, when the mitochondrial damage is below a critical threshold and there is no sufficient membrane potential for the fusion processes, mitophagy appears to eliminate the damaged network [51]. This pathway is preceded by fission mechanisms and mitochondrial fragmentation [52]. Our results together with those described above suggest that Medox anthocyanins are strong protectors of mitochondrial function.

In conclusion, we report that Medox anthocyanins ameliorate the mitochondrial dysfunction caused by A $\beta$  overproduction or rotenone inhibition of the mitochondrial complex I. Our results suggest that Medox can contribute in normalizing the mitochondrial fusion/fission processes. Thus promoting the distribution of mitochondrial components for the maintenance of energy output and cell viability. Further studies are needed to understand the impact of anthocyanins on the coordination of mitochondrial fusion/fission, mitophagy and mitogenesis pathways as well as the clinical implication in the prevention of neurodegenerative processes as AD.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbadis.2016.08.002>.

## Conflict of interest

None of the authors have a personal or financial conflict of interest.

## Transparency document

The Transparency document associated with this article can be found, in online version.

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