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A Specific Combination of Nutraceutical Ingredients exerts cytoprotective effects in human cholinergic neurons.

--Manuscript Draft--

Manuscript Number:	PHANU-D-22-00175R2
Article Type:	Full Length Article
Keywords:	brain aging; vitamin E; folic acid; Magnesium; Omega-3
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Manuscript Region of Origin:	Europe
Abstract:	Backgroud: Brain aging is associated with an excessive reactive oxygen species (ROS) formation that causes cell injury through proteins oxidation and DNA damage. These changes have been identified as contributing factors in age-related memory decline. In this sense, treatments able to protect central nervous system (CNS) from oxidative stress and to sustain membrane plasticity, may represent new candidates to counter the development of aging effects. Several studies have indicated vitamin E, folic acid, magnesium and omega-3 as nutraceuticals protecting CNS from oxidative stress. Methods: A specific association of these active nutrients was tested in human cholinergic neurons, chosen as a cellular model related to learning and memory processes. Cortisol was used as an oxidative stress insult to explore the beneficial properties of the nutraceuticals. Results: In summary, the specific ratio of active ingredients in the above selected food supplement prevented the decrease in ATP content and in cell viability exerted by cortisol. At the same time, it prevented ROS formation, DNA damage, autophagy processes and decrease in the expression of cellular well-being genes induced by cell treatment with cortisol. The effects on ATP content, ROS formation and cellular viability were evidenced when the nutraceutical mix when administered following cortisol treatment, too. Notably, these peculiar evidences were significantly higher with respect to those elicited by the single components of the food supplement. Conclusions: Overall, these results confirm the beneficial effects of the simultaneous administration of vitamin E, folic acid, magnesium and omega-3.
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October 6, 2022

Pharmanutrition

Dear Editor,

Please find enclosed the revised version of our manuscript entitled "A Specific Combination of Nutraceutical Ingredients exerts cytoprotective effects in human cholinergic neurons" that we submit for consideration to this Journal.

In this second revision, the abstract was modified and the manuscript carefully revised accordingly to the Guide for Authors.

We do hope that the manuscript can be considered for publication in Your Prestigious Journal.

Looking forward to hearing from you soon

Sincerely,

Claudia Martini and Simona Daniele

Comments from the Editors and Reviewers:

Please correctly format the Abstract and slowly and carefully read the Guide for Authors.

The abstract was modified and the manuscript revised accordingly to the Guide for Authors.

Reviewer #1: The authors have considered all comments and suggestions, so the present paper is suitable to publish in this journal.

Reviewer #2: The authors have meticulously addressed all issues raised by the original submission in an adequate manner. I think this paper is interesting and that the authors improve the composition of the discussion and the general quality of the manuscript.

We thank the Reviewers for their comments.

A Specific Combination of Nutraceutical Ingredients exerts cytoprotective effects in human cholinergic neurons.

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Abstract

Backgroud: Brain aging is associated with an excessive reactive oxygen species (ROS) formation that causes cell injury through proteins oxidation and DNA damage. These changes have been identified as contributing factors in age-related memory decline. In this sense, treatments able to protect central nervous system (CNS) from oxidative stress and to sustain membrane plasticity, may represent new candidates to counter the development of aging effects. Several studies have indicated vitamin E, folic acid, magnesium and omega-3 as nutraceuticals protecting CNS from oxidative stress.

Methods: A specific association of these active nutrients was tested in human cholinergic neurons, chosen as a cellular model related to learning and memory processes. Cortisol was used as an oxidative stress insult to explore the beneficial properties of the nutraceuticals.

Results: In summary, the specific ratio of active ingredients in the above selected food supplement prevented the decrease in ATP content and in cell viability exerted by cortisol. At the same time, it prevented ROS formation, DNA damage, autophagy processes and decrease in the expression of cellular well-being genes induced by cell treatment with cortisol. The effects on ATP content, ROS formation and cellular viability were evidenced when the nutraceutical mix when administered following cortisol treatment, too. Notably, these peculiar evidences were significantly higher with respect to those elicited by the single components of the food supplement.

Conclusions: Overall, these results confirm the beneficial effects of the simultaneous administration of vitamin E, folic acid, magnesium and omega-3.

Keywords: brain aging; vitamin E; folic acid; magnesium; omega-3; neuroprotection.

Abbreviations: 7-AAD, amino-actinomycin D; BDNF, brain-derived neurotrophic factor; bFGF basic fibroblast growth factor; ChAT, choline acetyltransferase; CNS, central nervous system; CORT, cortisol; CREB, cAMP response element-binding protein; DHA, docosahexaenoic acid; EGF, epidermal growth factor; EPA, eicosapentaenoic acid; FA, folic acid; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; H2DCFDA, fluorogenic dye 2′,7′-dichlorofluorescin diacetate; Keap1, kelch-like ECH-associated protein 1; LC3β, microtubule-associated protein light chain-3; Nrf2, nuclear factor-like 2; NSC, neural stem cells; PBS, phosphate buffered saline; PRG-1, plasticity-related gene 1; PUFA, polyunsaturated fatty acids; RA, retinoic acid; ROS, reactive oxygen species; SIRT-1, sirtuin.

1. Introduction

Brain aging has been associated with decline of biological functions, progressive memory loss, problems of coordination and difficulty in concentration [1]. During life, neuronal defense mechanisms decrease, leaving neurons exposed to free radical damage, protein accumulation and decreased neurogenesis [2, 3, 4]. Moreover, the reduction of the antioxidant defenses and the presence of high blood glucocorticoid levels [5], which characterize aging, could lead to an excessive reactive oxygen species (ROS) formation, causing inflammation through lipid peroxidation, glycosylation or oxidation of proteins and DNA damage [6]. Nutraceutical compounds can exert neuroprotective effects by regulating energy metabolism, neuro-oxidative stress, neuroinflammation and by improving neurogenesis. Herein, the nutraceutical combination used is a food supplement formulation containing a specific amount in a fixed ratio of active ingredients, including folic acid, vitamin E, magnesium and omega-3, which are essential to obtain the desired physiological balance [7]. Folic acid is an important vitamin for neuronal development [8] and for DNA stability. In fact, folate acts as a donor of methyl groups leading to the formation of S-adenosylmethionine which regulates DNA transcription and gene stability [9]. At the same time, vitamin E is a lipid-soluble antioxidant vitamin capable of blocking radical chain reactions and regulating signal transduction [10]. Another critical component for the maintenance of CNS functions is magnesium. Indeed, it is the second most important and abundant cation in cells, and it is essential for many physiological processes. Maintaining the appropriate magnesium concentrations is necessary for cell growth, proliferation, differentiation, energy metabolism and death [11]. Omega-3 are essential unsaturated fatty acids (n-3 PUFAs) and they are crucial components of neuronal cell membranes; among these, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are pivotal for antioxidant defenses [12] and to preserve the correct membrane cell fluidity [13]. Actually, the maintenance of a correct cell membrane plasticity allows to obtain a good cellular communication and to counteract memory and learning deficits related to the changes in neuronal and synaptic density [14, 15]. In this sense, maintaining adequate levels of these fatty acids leads to a reversion of age-related synaptic plasticity changes, a reduction of inflammation and brain atrophy [16]. In this context, treatments able to reduce inflammation, to increase neurotropism and to protect CNS from oxidative stress, may represent new candidates to counteract aging development and neuro-psychological alterations [17]. Currently, treatments composed of one or two ingredients, among folic acid, vitamin E, magnesium and omega-3 have been studied, but all together (as a mixture), at specific concentrations, have not been assessed yet. Although present in specific food sources not always easy to get, these nutraceutical compounds can be provided also through a healthy and balanced diet. In fact, n-3 PUFAs such as EPA and DHA are contained in fatty cold-water fishes (mackerel, sardine, herring, tuna, and salmon), fish oil, seafood, and krill oil [18, 19, 20]. The major dietary sources of vitamin E are vegetable oils (e.g., olive, coconut, sunflower, palm, soybean), whole grains, leafy vegetables, and nuts [21, 22, 23], while magnesium is taken frequently through dietary sources such as green leafy vegetables, cocoa, almonds, nuts, unground grains, whole seeds, legumes, and at lower concentrations through fruit, meat (chicken, pork), and fish [24, 25]. Finally, folic acid is the synthetic form of folates, used in supplements or fortified foods, while folates are provided naturally by eating green leafy vegetables, yeast, liver, eggs, fermented products (e.g., cheese, yogurt), legumes, and some fruits [26, 27]. In the present work, the active ingredients were combined according to a peculiar percentage reported in Libretto®/Primus® and tested in human cholinergic neuronal-like cells (i.e., differentiated SHSY-5Y cells) for their abilities to protect neurons against age-related cellular alterations [28].

For the purpose of the study, the SH-SY5Y culture system has been chosen, which is a convenient neuronal model. This model has the potential to elaborate human/primate-specific transcription networks and pathways related to human cognitive disorders [29], which has been already frequently used to explore neurodegeneration, oxidative stress, and psychological disorders [30]. In particular, recent literature has reported that SHSY-5Y cell differentiation with retinoic acid (RA) and brain-derived neurotrophic factor (BDNF), as in the present paper, induces the expression of acetylcholinesterase and choline acetyltransferase enzymatic activities and it has been proposed as a suitable model of cholinergic neurons to study cellular processes associated to aging and neurodegeneration [31].

2. Materials and Methods

2.1 Materials

cis-5,8,11,14,17-eicosapentaenoic acid (EPA), cis-4,7,10,13,16,19-docosahexaenoic acid (DHA), folic acid (FA), D-alpha-tocopherol polyethylene glycol (vitamin E), magnesium carbonate (Mg^{2+}) hydrocortisone for cortisol (CORT), retinoic acid (RA) and brain-derived neurotrophic factor (BDNF) were purchased from Sigma Aldrich. All other reagents were obtained from standard commercial sources. In this paper, the complete ingredients mix (Libretto®/Primus®, Angelini Pharma S.p.A) "nutraceutical combination" or as a "nutraceutical mix" and it was used in two set of dilutions: MIX [1'] refers to 8 μM EPA, 3.12 μM DHA, 4.5 nM folic acid, 52 nM vitamin E and 2.8 μM magnesium while MIX [1''] refers to 0.8 μM EPA, 312 nM DHA, 0.45 nM folic acid, 5.2 nM vitamin E and 280 nM magnesium. EPA, DHA and folic acid were diluted to different concentrations of stock solutions by 100% DMSO; otherwise, vitamin E and magnesium were diluted by water. All subsequent dilutions were carried out in water, therefore in the final composition of the nutraceutical, there are negligible amounts of each solvent.

2.2 Cell cultures differentiation and induction of stress

SH-SY5Y neuroblastoma cell line was cultured in a complete medium consisting of DMEM, 10% fetal bovine serum (FBS), and 2 mM L-glutamine, at 37 °C in 5% CO_2. For cholinergic neuronal differentiation, SH-SY5Y were cultured in DMEM reducing FBS concentration to 2% and supplementing with 10 μM RA for 7 days. This treatment was replaced every 2 days. On the fourth day of differentiation 50 ng/mL of BDNF were added [31]. To set up the stress model, an MTS assay was performed and hydrocortisone (water soluble, H0396, Sigma Aldrich, Milan, Italy) at 250 μM for 24 h was used on human neurons. H9-derived Neural Stem Cells (NSCs) were purchased from GIBCO (Life Technologies, Milan, Italy). They were cultured in complete medium consisting of KnockOut™D-MEM/F-12 with StemPro® Neural Supplement, 20 ng/ml of basic fibroblast growth factor (bFGF, Life Technologies, Milan, Italy), 20 ng/ml of epidermal growth factor (EGF, Life Technologies, Milan, Italy), and 2 mM L-glutamine at 37 °C in 5% CO_2.

2.3 Immunohistochemistry

Cell differentiation was evaluated using immunofluorescence. Cells were washed with phosphate buffered saline (PBS), fixed with paraformaldehyde (4% v/v) for 20 min at room temperature (RT), and permeabilized with 0.1% Triton X-100-supplemented PBS. Nonspecific binding was blocked with 2.5% BSA for 1 h at RT. After PBS washes, cells were incubated with anti-ChAT (abcam, ab178850) antibody, overnight at 4°C. Subsequently, a secondary antibody anti-rabbit Alexa Fluor® (Invitrogen, 1:500) was used, for 45 min at RT [32]. Ten microscopic fields (× 60 magnification) were selected randomly from each of three independent experiments (n= 3). The images were obtained with a Zeiss fluorescence microscope equipped with ApoTome technology (Zeiss Microscopy, Jena, Germany) and analyzed with ImageJ software. To obtain a quantitative indication of differentiated cells, Alexa Fluor®-positive cells were counted, and the percentage of ChAT-positive cells was calculated by using DAPI labelling to obtain the total cell number. Subsequently, with the ImageJ program the relative fluorescence was calculated in control and differentiated cells. DAPI labelling was used to normalize fluorescence intensity to the same number of cells. The data were expressed as fold change of fluorescence intensity vs control cells.

2.4 Apoptosis Assessment

SH-SY5Y neuroblastoma cell line was seeded in 6-multiwell plates (10000 cells/ well or 5.000 cell/ml). After differentiation into cholinergic neurons (1 week), cells were treated with nutraceutical combination at [1'] and [1''] concentrations for 24 h and subsequently with hydrocortisone or sodium azide (NaN_3) for an additional 24 h. After treatment, early and late apoptotic SH-SY5Y were estimated by Muse Apo Assays (Merck-

Millipore) as previously reported [33]. Apoptotic and dead cells were distinguished using the annexin V conjugated with fluorescein isothiocyanate (FITC) and amino-actinomycin D (7-AAD) [33].

2.5 ATP and ADP assay

SH-SY5Y neuroblastoma cell line was seeded in 96-multiwell plates (5000 cells/ well or 50.000 cells/ml). After differentiation into cholinergic neurons (1 week), cells were treated with 8 μM EPA, 3.12 μM DHA, 4.5 nM folic acid, 52 nM vitamin E or 2.8 μM magnesium, or the nutraceutical combination at [1''] and [1'] concentrations for 24 h. Following incubation time, cells were incubated with hydrocortisone for an additional 24 h. In contrast, in "post-treatment experiments", cells were incubated with hydrocortisone and then challenged with the nutraceutical combination for 24 h. At the end, the amount of ATP and ADP were estimated using a bioluminescence assay kit, according to the manufacturer's instruction (ADP/ATP Ratio Assay Kit, Abcam, Milan, Italy) [33]. The luminescence data were reported as the ATP/ADP ratio vs control cells set to 100%.

2.6 Cell proliferation assay

SH-SY5Y neuroblastoma cell line was seeded in 96-multiwell plates (5000 cells/ well or 50.000 cells /ml) and differentiated into cholinergic neurons, as described above. Moreover, H9-derived NSCs were used in parallel experiments to investigate the effects of the supplement on stem cells viability. SH-SY5Y neuroblastoma cell line was treated with 8 μM EPA, 3.12 μM DHA, 4.5 nM folic acid, 52 nM vitamin E or 2.8 μM magnesium, or the nutraceutical combination at [1''] and [1'] concentrations for 24 h. Following incubation time, cells were incubated with hydrocortisone for an additional 24 h. In contrast, in "post-treatment experiments", cells were incubated with hydrocortisone and then challenged with the nutraceutical combination for 24 h. At the end of treatments, cell proliferation was determined using the MTS assay according to the manufacturer's instruction. Within an experiment, each condition was analyzed in triplicate, and each experiment was performed at least three times. The results were calculated by subtracting the mean background from the values obtained from each test condition. Final data were reported as the fold change from control cells set to 100% [34].

2.7 Western blot analysis

SH-SY5Y neuroblastoma cell line was seeded in 6-multiwell plates (10000 cells/well or 5.000 cells/ml). After differentiation into cholinergic neurons (1 week), cells were treated with nutraceutical combination at [1''] and [1'] concentrations for 24 h. Following incubation time, cells were exposed to 250 μM of hydrocortisone for an additional 24 h. At the end of the treatment period, cells were collected and then were lysed for 2 hours at 4 °C in RIPA buffer (9.1 mM NaH_2PO_4, 1.7 mM Na_2HPO_4, 150 mM NaCl, pH 7.4, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1 % SDS, and a protease-inhibitor cocktail). Equal quantities of the cell extracts (50 μg of protein) were diluted in Laemmli solution, resolved using SDS-PAGE (8.5%), transferred to PVDF membranes, and probed overnight at 4 °C using an antibody anti-choline acetyltransferase (ChAT, ab178850 abcam, 1:200) anti-microtubule-associated protein light chain-3 (LC3β, sc-28266 Santa Cruz Biotechnology, 1:100) [35] or anti-oxidative proteins nuclear factor-like 2 (Nrf2, ab137550 abcam, 1:200) or kelch-like ECH-associated protein 1 (Keap1, AV38981 Sigma-Aldrich, 1:200). The primary antibody was detected using the appropriate peroxidase-conjugated secondary antibody, which was then detected using a chemiluminescent substrate (ECL, Perkin Elmer). Densitometric analysis of the immunoreactive bands was performed using Image Lab Software. The densitometric analysis was performed taking into consideration both bands relating to the two different forms of LC3β. The data are reported as the fold change from control cells set to 100%. All western blots were performed with the ChemiDoc XRS+Gel Imaging System (Biorad, Milan, Italy), which includes gel activation and acquisition of the total proteins imprinted in the PVDF. This "stain-free protein normalization" allows the normalization of bands to total protein in blots, thus eliminating the need for housekeeping proteins.

2.9 ROS assay

SH-SY5Y neuroblastoma cell line was seeded in 96-multiwell black plates (5000 cells/ well). After differentiation into cholinergic neurons (1 week), cells were treated with 8 μM EPA, 3.12 μM DHA, 4.5 nM folic acid, 52 nM vitamin E or 2.8 μM magnesium, or the nutraceutical combination at [1''] and [1'] concentrations for 24 h. Following incubation time, cells were exposed to 250 μM of hydrocortisone for an additional 24 h. In contrast, in "post-treatment experiments", cells were incubated with hydrocortisone and then challenged with the nutraceutical combination for 24 h. ROS activity was determined using the fluorogenic dye 2′,7′-dichlorofluorescin diacetate (H2DCFDA, Molecular Probes, Invitrogen). Following incubation with treatments, cells were washed and a solution of PBS + glucose 10 mM with 50 μM of H2DCFDA was added in the dark at 37 °C for 30 minutes. The fluorescence intensity (excitation 485 nm and emission 520 nm) was normalized based on the number of cells stained with crystal violet [33]. Data were reported as the fold change from control cells set to 100%.

2.10 Quantification of DNA damage

SH-SY5Y neuroblastoma cell line was seeded in 96-multiwell plates (5000 cells/ well). After differentiation into cholinergic neurons (1 week), cells were treated with nutraceutical combination at [1''] and [1'] concentrations for 24 h; at the end of treatments, cells were incubated with 250 μM of hydrocortisone for an additional 24 h. Following, cells were lysed, and equal amounts of protein were incubated in precoated wells. The DNA damage was estimated using an IR assay kit (Human H2A.X phospho S139 ELISA Kit, Abcam, Milano, Italy) according to manufacturer's instructions [33]. The data are reported as fold change over control cells (100%).

2.11 Evaluation of membrane fluidity

SH-SY5Y neuroblastoma cell line was seeded in 96-multiwell plates (5000 cells/ well). After differentiation into cholinergic neurons (1 week), cells were treated with nutraceutical combination at [1''] and [1'] concentrations for 24 h. Following incubation time, cells were exposed to 250 μM of hydrocortisone for an additional 24 h. Membrane fluidity was measured by flow cytometry using a fluorescent labelling method according to the manufacturer's protocol (Membrane Fluidity kit, Abcam, Milan, Italy). The EnSight™ multimode plate reader, measuring the excitation at 350 nm and the emission at 470 nm (excimer) and 370 nm (monomer), was used for analyzing the fluorescence intensity of labeled cells. Quantitative monitoring of the membrane fluidity was obtained by measuring the ratio of monomer to excimer fluorescence [36]. The data are reported as fold change over control cells (100%).

2.12 RNA extraction and real-time PCR analysis

SH-SY5Y neuroblastoma cell line was seeded in 6-multiwell plates (10.000 cells/ well or 5.000 cells/ml) and differentiated into cholinergic neurons. In parallel experiments, H9-derived NSCs were used to investigate the effects of the supplement on stem cells. Both cell lines were treated with nutraceutical combination at [1''] and [1'] concentrations for 24 h; after the incubation time, cells were exposed to 250 μM of hydrocortisone for an additional 24 h. At the end of treatments, cells were collected, and total RNA was extracted using Rneasy® Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The purity of the RNA samples was determined by measuring the absorbance at 260:280 nm and cDNA synthesis was performed with 500 ng of RNA using i-Script cDNA synthesis kit (BioRad, Hercules, USA). Primers used for RT-PCR were designed in intron/exon boundaries to make sure that products did not include genomic DNA [34]. RT-PCR reactions consisted of 10 μL Fluocycle® II SYBR® (Euroclone, Milan, Italy), 0.6 μL of both 10 μM forward and reverse primers, 5 μL cDNA, and 3.8 μL of H_2O. All reactions were performed for 40 cycles using the following temperature profiles: 98 °C for 30 s (initial denaturation); T °C (see Table 1) for 30 s (annealing); and 72 °C for 3 s (extension) [36]. β-actin was used as the housekeeping gene. PCR specificity was determined

by both the melting curve analysis and gel electrophoresis. The data were reported as fold change over control cells set to 1.

Gene	Primer nucleotide sequences	Product size (base pairs)	Annealing temperature
BDNF	FOR: 5'-TACATTTGTATGTTGTGAAGATGTTT-3' REV: 5'-CCTCTTTTCAGAAAAATTCAGGA-3'	131	56°
SIRT1	FOR: 5'-CCTGGACAATTCCAGCCATC-3' REV: 5'-TTCATGATAGCAAGCGGTTCAT-3'	272	66°
CREB	FOR: 5'-AAGCTGAAAGTCAACAAATGACA-3' REV: 5'-CCTCTTTTCAGAAAAATTCAGGA-3'	240	52°
PRG-1	FOR: 5'-CCCGCTCAGGGAATAGCTG-3' REV: 5'-GCTAACCACCGATGATGCCA-3'	156	54°
B-actin	FOR: 5'-GCACTCTTCCAGCCTTCCTTCC-3' REV: 5'-GAGCCGCCGATCCACACG-3'	54	55°

Table 1. Nucleotide sequences and annealing temperature of the primers utilized in PCR.

2.13 COX-2 assay

SH-SY5Y neuroblastoma cell line was seeded in 96-multiwell plates (5000 cells/ well). After differentiation into cholinergic neurons (1 week), cells were treated with nutraceutical combination at [1''] and [1'] concentrations for 24 h. Following incubation time, cells were exposed to 250 µM of hydrocortisone for an additional 24 h. The amount of COX-2 in supernatant was estimated using an enzyme-linked immunosorbent assay (Human Cox-2 ELISA kit, Sigma Aldrich) according to manufacturer's instructions. Data were reported as a fold change over control cells set to 100%.

2.14 IL-6 and IL-8 assay

SH-SY5Y neuroblastoma cell line was seeded in 96-multiwell plates (5000 cells/ well). After differentiation into cholinergic neurons (1 week), cells were treated with nutraceutical combination at [1''] and [1'] concentrations for 24 h. Following incubation time, cells were exposed to 250 µM of hydrocortisone for an additional 24 h. The interleukins content in the supernatant was measured using enzyme-linked immunosorbent assay (ELISA) kits (Cloud-Clone Corp., Katy, Texas, United States: SEA079Hu for IL-6, SEA080Hu for IL-8) following the manufacturers' instructions. Briefly, 100 µL of supernatant was added into the appropriate wells and incubated for 1 hour at 37 °C. After incubation time, 100 µL of primary antibody were added for 1 hour at 37 °C. After extensive washes, 100 µL of secondary antibody were incubated for 30 minutes at 37 °C and then, the substrate solution was added to each well, leaving the color to develop for 10-20 min at 37 °C. Absorbance was measured at 450 nm and a standard curve was obtained with standards kit.

2.15 Statistical Analysis

Data analysis was performed using one-way analysis of variance (ANOVA) with "Bonferroni's Multiple Comparison Test". $p<0.05$ was considered statistically significant.

3. Results

3.1. Differentiation of SHSY-5Y human neuroblastoma cell line into cholinergic neurons

Aging is characterized by loss of some cholinergic system functions [37]. To investigate the effect of nutraceutical combination, human neuroblastoma cell line SHSY-5Y was differentiated into a cholinergic neuronal system with retinoic acid (RA) and brain-derived neurotrophic factor (BDNF) for seven days [31]. To confirm SH-SY5Y differentiation in cells with characteristics of cholinergic neurons, the expression of choline acetyltransferase (ChAT) enzyme was evaluated by Western blot analysis. The induction of differentiation led to an increase in the expression of protein involved in the production of acetylcholine, demonstrating the acquisition of typical characteristics of cholinergic neurons (Supplementary Figure 1 a,b). To confirm the results obtained with western blot analysis, an immunostaining of ChAT was performed (Supplementary Figure 1 c,d). In control undifferentiated cells, 25±3% of total cells were positive to ChAT, confirming the basal expression of the selected protein in these neuronal-like cells [31]. Following cell treatment, 80±6% of cells expressed ChAT, thus demonstrating that RA plus BDNF significantly induced SHSY-5Y differentiation into cholinergic neurons. Quantification of the relative fluorescence intensity, normalized to the same cell number, showed that differentiation induced a 1.76-fold increase in fluorescence intensity (Supplementary Figure 1d).

3.2. Effect of cortisol on cell viability and evaluation of cortisol induced apoptosis

Assuming that an increase in glucocorticoid levels has been observed with aging [5] and that chronic and non-physiological levels of cortisol induce an increase in oxidative stress leading to the formation of reactive oxygen species (ROS) [38], a cortisol stress model [28] was developed to evaluate and to corroborate nutraceutical mix neuroprotective effects. For this purpose, cells were treated with two different concentrations (250 µM and 500 µM) of cortisol (CORT) for different times (4,8,16,24 h) and then, cell viability was assessed (Figure 1a). The concentration of 500 µM caused a reduction in cell viability of about 55%, almost mimicking acute stress. The lowest concentration (250 µM) produced a less marked reduction of cell viability, also at longer incubation times. Based on the obtained results, and in order to simulate a daily chronic physiological stress, this lower concentration for the longer time (24 h) was chosen.
In order to study the effect of cortisol on cellular conditions, apoptotic staining was performed. In parallel, sodium azide, an inhibitor of the fourth mitochondrial complex with apoptotic effect, was used to compare the behavior of cortisol [39, 40]. As Figure 1 (panel b) shown, the trend of cortisol (24.57% in early apoptosis and 12.36% in late apoptosis) appeared very similar to sodium azide (23.57% in early apoptosis and 11.23% in late apoptosis), demonstrating to have analogous effects. In supplementary materials (Supplementary Figure 2 a,b), we demonstrated that both mixes were able to protect cells from apoptosis damage cortisol-induced.

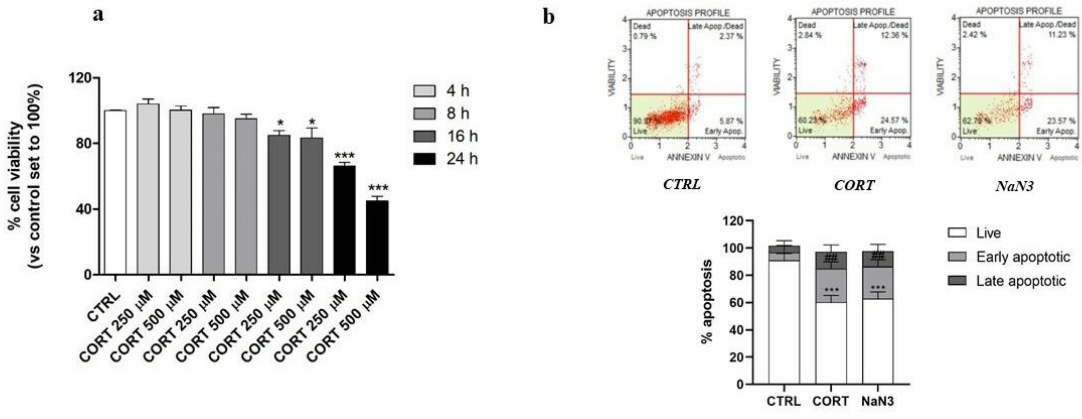

Figure 1. Stress model with cortisol. (a) The SH-SY5Y cell line was differentiated into cholinergic neurons and after they were treated with cortisol at concentrations of 250 µM and 500 µM for 4, 8, 16, and 24 h. Following incubation, cellular viability was measured by MTS assay. Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni's post-test: * $p<0.05$, *** $p<0.001$. (b) The SH-SY5Y cell line was differentiated into cholinergic neurons and after, they were treated with cortisol at concentration of 250 µM or with sodium azide at concentration of 3 mM for 24 h. Following incubation, cellular apoptosis was measured by Muse Apo Assays (Merck-Millipore) as previously reported [33]. The data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni's post-test: *** $p<0.001$ and $^{\#\#}$ $p<0.01$ versus control of live cells and early apoptotic cells, respectively.

3.3. Assessment of suitable concentrations of the nutraceutical combination

In order to establish the safe and non-toxic concentrations of nutraceutical mix, cellular viability was tested for each nutraceutical compound. At the same time, particular concentrations were chosen to maintain an exact and specific proportion between compounds within the mix (Figure 2). As shown in Figure 2 the mix containing 8 µM EPA, 3.12 µM DHA, 4.5 nM folic acid, 52 nM vitamin E and 2.8 µM magnesium, here indicated as mix [1'], was initially chosen as single ingredients that did not reduce cell viability by more than 20%. Subsequently, the mix was further diluted (ten-fold reduction) and used as final concentrations of active ingredients (0.8 µM EPA, 312 nM DHA, 0.45 nM folic acid, 5.2 nM vitamin E and 280 nM magnesium). Here, this second mix is indicated as mix [1''] and it was chosen to assess if the beneficial effects can be maintained even at lower concentrations too (Figure 2).

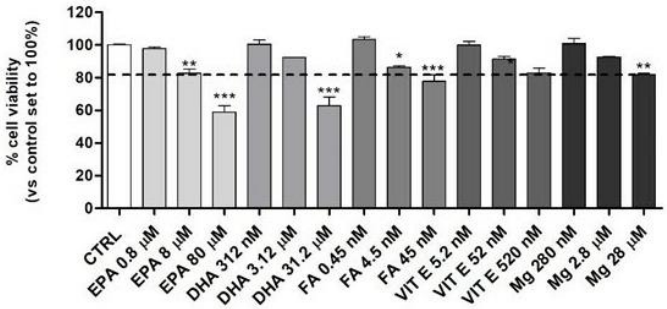

Figure 2. Assessment of mix concentrations. The SH-SY5Y cell line was differentiated into cholinergic neurons, and, after, they were treated with different dilution of active ingredients of nutraceutical combination. Then, two sets of concentrations were chosen, indicated as: mix [1''] and mix [1']. Following incubation, cellular viability was measured by MTS assay. Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni's post-test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs control.

3.4. Effect of the nutraceutical combination on ATP/ADP ratio and cell viability

In light of recent publications and insights on the action of glucocorticoid receptors at mitochondrial level [41], we considered appropriate to examine the cellular energy balance as an index of mitochondrial dysfunction and mitochondrial protective potential of nutraceutical mix. As depicted in Figure 3, in the absence of cortisol, the mix did not cause any significant change in ATP levels, both at [1''] and [1'] concentrations. As expected, the action of cortisol on glucocorticoid receptors resulted in energy damage; while, in contrast, nutraceutical mixes were able to prevent the cortisol-induced imbalance of ATP/ADP ratio, suggesting an involvement of supplements in restoring ATP levels (Figure 3a). Notably, similar results were obtained when the mix was administered following cortisol (Figure 3a), thus suggesting that the nutraceutical combination can rescue the metabolic alterations induced by cellular stress, too. The effects displayed by the nutraceutical mix on

ATP/ADP content were significantly higher with respect to those elicited by the single component, i.e., EPA/DHA, folic acid, vitamin E or magnesium (Supplementary Figure 3a).
Due to the ATP reduction, an assay of cell viability was assessed. In the absence of cortisol, the mix did not cause reduction in cell viability, neither at [1’’] and [1’] concentrations; as expected, challenging cells with cortisol significantly decreased cell viability but this reduction was prevented by challenging cells with both mix (Figure 3b). Notably, a significant rescue in cortisol-induced decrease in cellular viability was obtained when the mix was administered following cortisol (Figure 3b). The cytoprotective effects elicited by the nutraceutical combination did not significantly differ from those obtained by testing the single components alone, i.e., EPA/DHA, folic acid, vitamin E or magnesium (Supplementary Figure 3b).
In order to study the effect of nutraceutical mix on the viability of a stem cell line, H9-derived NSCs were chosen. The mix displayed similar effects in this cell line: specifically, in the absence of cortisol, it did not cause reduction in cell viability, either at [1 "] or [1 '] concentrations; as expected, challenging cells with cortisol significantly decreased NSC viability (Supplementary Figure 4). The decrease in NSC viability was prevented by nutraceutical mix (Supplementary Figure 4). These data evidenced a cytoprotective effect of this nutraceutical combination in cholinergic neurons and in neural stem cells.

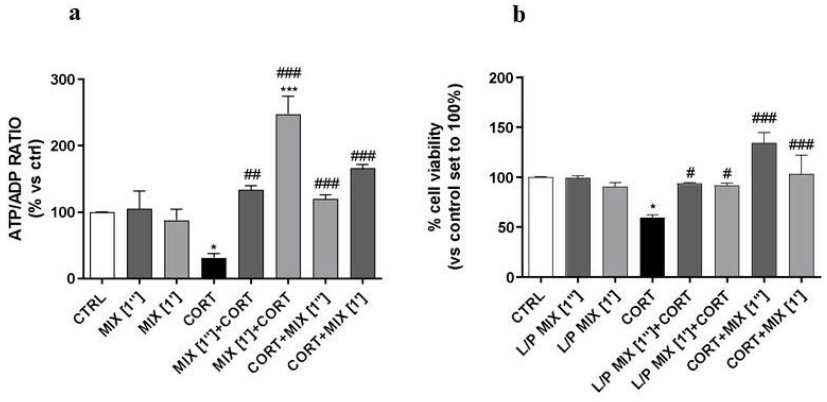

Figure 3. (a-b) Evaluation of the ATP/ADP ratio (energy balance) and cell viability. Following differentiation into cholinergic neurons, cells were treated with nutraceutical mix at [1’’] and [1’] concentrations, for 24 h and for an additional 24 h with cortisol at 250 μM (MIX[1’’]+CORT or MIX[1’]+CORT). In post-treatment experiments (CORT+MIX[1’’] or CORT+MIX[1’]), differentiated cells were challenged with 250 μM cortisol for 24 h, and then with the nutraceutical mix at [1’’] and [1’] for an additional 24 h. The ATP/ADP ratio was evaluated by specific fluorometric assay (a), and cellular viability was measured by MTS assay (b). Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni’s post-test: * $p<0.05$, *** $p<0.001$ vs control; $^{\#}$ $p<0.05$; $^{\#\#}$ $p<0.01$, $^{\#\#\#}$ $p<0.001$ vs cortisol.

3.5. Effect of nutraceutical combination treatment on autophagy pathway

Autophagy is a mechanism that allows the degradation and recycling of cellular components. During this process, the damaged cytoplasmic constituents are isolated from the rest of the cell within a double-membrane vesicle known as autophagosome. The formation of the autophagosome occurs when the cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form the conjugated form LC3-phosphatidylethanolamine (LC3-II). At this stage, the autophagosome membrane fuses with that of a lysosome and the contents are degraded and recycled [42]. Since ATP reduction activated a serine/threonine kinase (AMPK) [43], triggering autophagy processes [44], the influence of the food supplements on autophagy phenomena was investigated. As reported in literature autophagic activity at the neuronal level is constitutively and physiologically higher than in other districts [45]. For this reason, in the absence of cortisol, both bands related to LC3β-I and LC3β-II protein were evident, validating the constitutive autophagic hypothesis of neuronal cells (Figure 4a). The increase in cortisol-induced autophagy was prevented by the following treatment with nutraceutical mix at both concentrations (Figure 4b). This signal may suggest a restoration of cellular balance and a consequent reduction of autophagy processes.

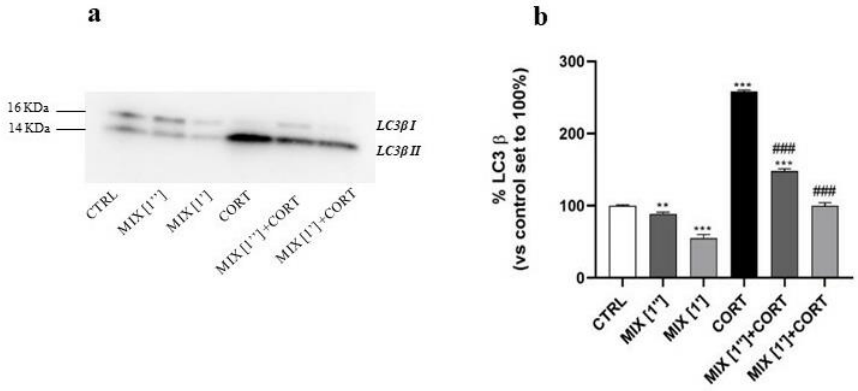

Figure 4. (a-b) Evaluation of autophagy induction: LC3β. Following differentiation into cholinergic neurons, cells were treated with nutraceutical mix [1'’] and nutraceutical mix [1'] for 24 h and for an additional 24 h with cortisol at 250 μM. After incubation, cells were collected and lysed. Autophagic marker LC3 (I and II) levels were assessed by western blot analysis using the anti-LC3β antibody. A representative Western blotting is shown (a). Irrelevant parts of the gel image are deleted. Optical density was measured by Image Lab software (b). Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as percentage of untreated cells (control set to 100%). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni's post-test: ** $p<0.01$, *** $p<0.001$ vs control; $^{###}$ $p<0.001$ vs cortisol.

3.6. Effect of nutraceutical combination treatment on ROS levels

Prolonged exposure to stress determines an abnormal and continuous secretion of glucocorticoids, leading to oxidative damage in the CNS [38]. In the absence of cortisol, nutraceutical mix did not cause an increase in oxidative damage, at [1'’] and [1'] concentrations. As expected, cortisol treatment significantly increased ROS levels (Figure 5). Challenging cells with the mix for 24 h before oxidant damage significantly prevented ROS formation at both sets of concentrations (Figure 5 MIX[1'’]+CORT or MIX[1']+CORT). These data demonstrate that this nutraceutical combination protects against oxidative stress. Notably, similar results were obtained when the mix was administered following cortisol (Figure 5 CORT+MIX[1'’] or CORT+MIX[1']), thus suggesting that the nutraceutical combination can rescue ROS imbalance induced by cellular stress, too.

The effects displayed by the nutraceutical mix on ROS production were significantly higher with respect to those elicited by the single component, i.e., EPA/DHA, folic acid, vitamin E or magnesium (Supplementary Figure 5).

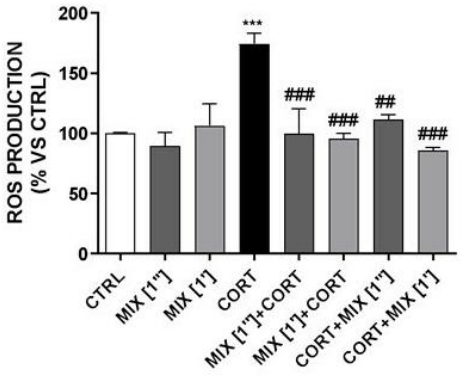

Figure 5. Evaluation of ROS levels. Following differentiation into cholinergic neurons, cells were treated with nutraceutical mix [1'’] and nutraceutical mix [1’] for 24 h and for an additional 24 h with cortisol at 250 µM (MIX[1'’]+CORT or MIX[1’]+CORT). In post-treatment experiments, differentiated cells were challenged with 250 µM cortisol for 24 h, and then with the nutraceutical mix at [1'’] and [1’] for an additional 24 h (CORT+MIX[1'’] or CORT+MIX[1’]). Oxidative stress levels were assessed by H2DCFDA fluorometric probe. Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni’s post-test: *** $p<0.001$ vs control; ## $p<0.01$, ### $p<0.001$ vs cortisol.

3.7. Effect of cortisol treatment on anti-oxidative proteins

Generally, the increase in oxidative stress determines an increase in cellular antioxidant defenses. Under acute stress conditions, Nrf2 dissociates from Keap1 and translocates into the nucleus where it may act as a transcription factor for antioxidant response genes [46]. In conditions of chronic stress, the Nrf2 pathway fails due to its degradation, depriving the cell of important antioxidant defenses [47].

To explore the effect of nutraceutical mix on antioxidant defenses, a western blot assay was performed to detect Nrf2 and Keap1 proteins. As depicted in Figure 6a,a’, cortisol treatment induced a significant decrease in Nrf2 proteins levels, respectively. In contrast, the mix, tested at both concentrations, demonstrated the ability to prevent Nfr2-decrease induced by cortisol.

These results demonstrate that cortisol mediates a reduction in the antioxidant activity of Nrf2-Keap1 complex, and that this nutraceutical combination is able to preserve the activity of Nrf2-Keap1 axis.

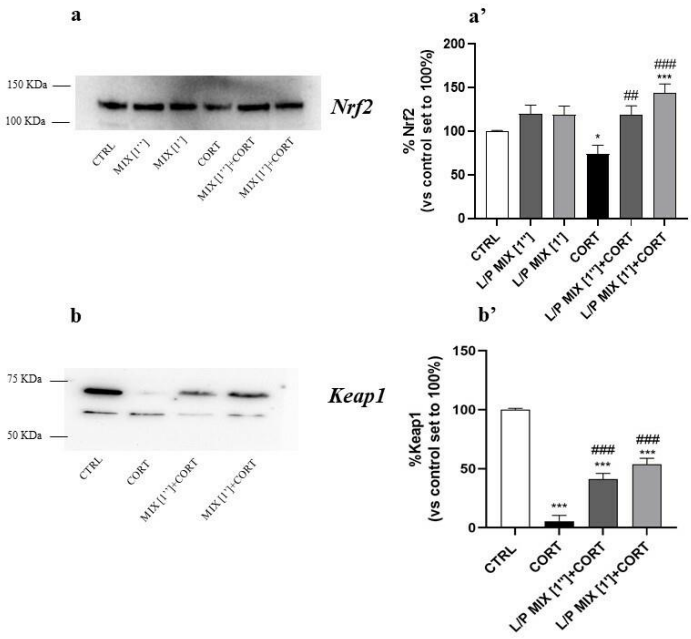

Figure 6. Evaluation of antioxidant protein levels. Following differentiation into cholinergic neurons, cells were treated with nutraceutical mix [1'’] and nutraceutical mix [1’] for 24 h and for an additional 24 h with cortisol at 250 µM. After incubation, cells were collected and lysed. Anti-oxidative proteins kelch-like ECH-associated protein 1 (Keap1) and nuclear factor-like 2 (Nrf2) levels were assessed by western blot analysis using the anti- Keap1 or anti- Nrf2 antibodies. A representative Western blotting is shown (a,b). Optical density was measured by Image Lab software (a’,b’). Data are the mean ± SEM of three different experiments and are reported as the percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni’s post-test: * $p<0.05$, *** $p<0.001$ vs control; $^{\#\#}$ $p<0.01$, $^{\#\#\#}$ $p<0.001$ vs cortisol.

3.8. Effect of the nutraceutical combination treatment on DNA damage

Prolonged stress leads to an increase in reactive oxygen species [38] which in turn are responsible for DNA damage [48]. Considering that this nutraceutical combination was able to reduce stress-induced oxidative damage, we next verified whether it could preserve human neurons from DNA damage. For this purpose, histone H2AX phosphorylation was evaluated as a marker of DNA damage [49]. As depicted in Figure 7, the mix was able to reduce histone H2AX phosphorylation at both sets of dilutions tested, demonstrating to have an intrinsic capacity in DNA protection. As expected, cortisol significantly increased histone H2AX phosphorylation; this increase was significantly contrasted with supplement mix at both sets of concentrations (Figure 7). Taken together, these results demonstrate that pre-treatment of human neurons with this nutraceutical combination reduces histone H2AX phosphorylation and prevents cortisol-induced DNA damage.

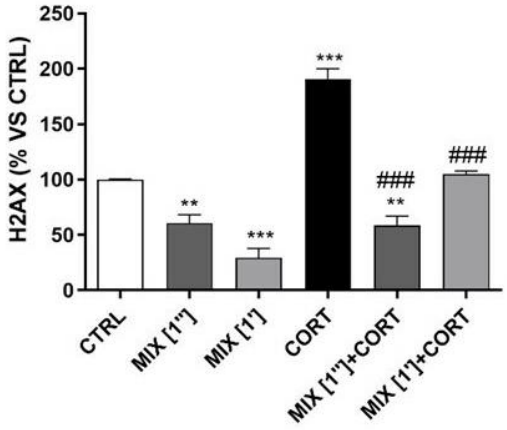

Figure 7. Evaluation of DNA damage. Following differentiation into cholinergic neurons, cells were treated with nutraceutical mix [1'’] and nutraceutical mix [1’] for 24 h and for an additional 24 h with cortisol at 250 µM. DNA damage was assessed by evaluation of histone H2AX phosphorylation levels. Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni’s post-test: ** $p<0.01$, *** $p<0.001$ vs control; ### $p<0.001$ vs cortisol.

3.9. Effect of the nutraceutical combination treatment on membrane fluidity

In the absence of antioxidant defenses, the predisposition in the brain to develop radical species [2] and oxidative stress, caused by non-physiological levels of cortisol [50], is responsible for the impairment of membrane fluidity. In this context, the effect of nutraceutical combination on membrane fluidity was analyzed. In the absence of cortisol, the mix had no influence on membrane fluidity; whereas, cortisol greatly reduced it, nutraceutical mix was able to prevent this reduction at [1'’] and [1’] concentrations (Figure 8), demonstrating its ability to protect the cell membrane from damage caused by cortisol-induced oxidative stress.

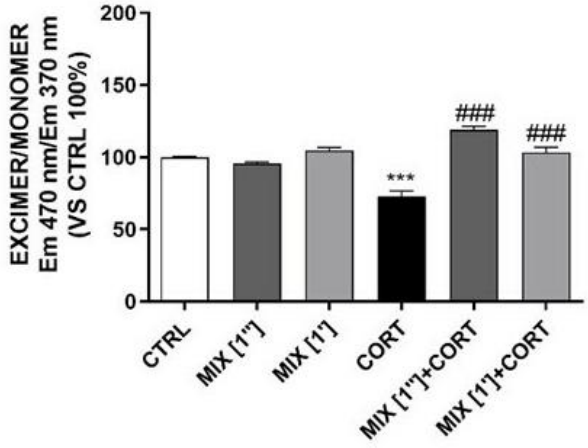

Figure 8. Evaluation of membrane fluidity. Following differentiation into cholinergic neurons, cells were treated with nutraceutical mix [1'’] and nutraceutical mix [1’] for 24 h and for an additional 24 h with cortisol at 250 µM. Membrane fluidity levels were assessed by fluorescence assay. Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni’s post-test: ** $p<0.01$ vs control; ### $p<0.001$ vs cortisol.

3.10. Effect of the nutraceutical combination treatment on aging-related genes

Alterations in the expression of genes such as brain-derived neurotrophic factor (BDNF) [51], cAMP response element-binding protein (CREB) [52], sirtuin (SIRT-1) [53] and plasticity-related gene 1 (PRG-1) have been related to different pathological mechanisms, including neuronal aging [54]. Challenging cholinergic neurons with the nutraceutical combination caused per se a significant enhancement in BDNF, SIRT1 and PRG-1 (Figure 9a,b,c,d). Cortisol decreased significantly the transcriptional levels of BDNF, SIRT1, CREB and PRG-1; the pre-treatment with the nutraceutical mix attenuated the cortisol-mediated decrease in the transcriptional levels of the examined genes, both at [1''] and [1'] concentrations (Figure 9a,b,c,d). These results may suggest that this nutraceutical combination effects on the induction of BDNF, CREB, SIRT-1 and PRG-1 gene expression may contribute to its cytoprotective effects in aged cholinergic neurons.

In order to study the effect of the mix on the induction of neurotrophic gene expression in a neural stem cell line, the H9-derived NSCs were used. Cortisol induced a significant reduction in the mRNA expressions of well-being-related factors (Supplementary Figure 6). Treatment with nutraceutical combination significantly prevented cortisol-induced effects, by reactivating the transcription of BDNF, CREB and SIRT-1 genes. The reduction in the expression of cortisol-induced genes, resulting in an increase in cellular well-being (Supplementary Figure 6).

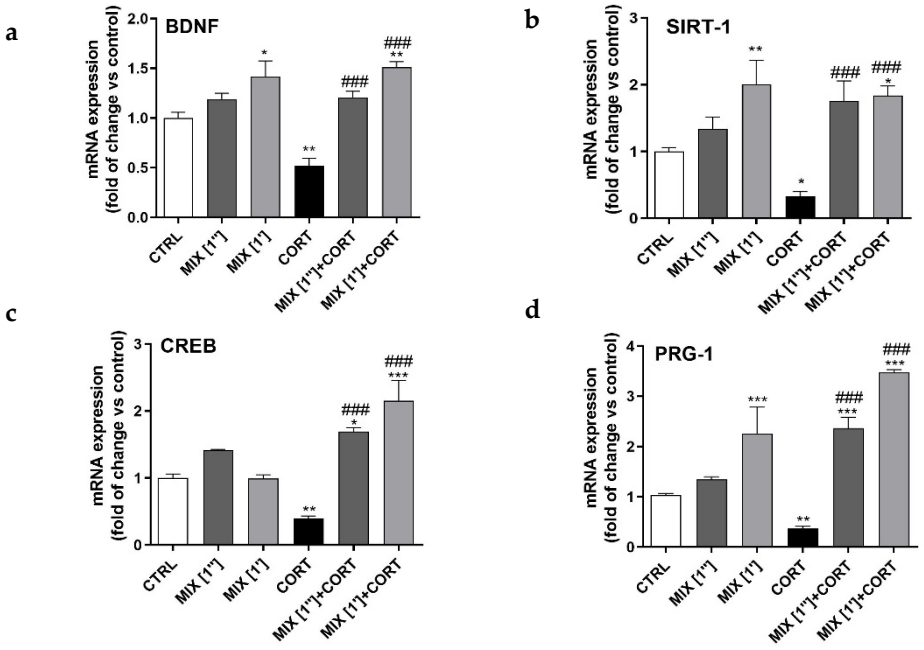

Figure 9. (a-d) Evaluation of genes expression related to well-being and neuronal plasticity. Following differentiation into cholinergic neurons, cells were treated with nutraceutical mix [1''] and nutraceutical mix [1'] for 24 h and for an additional 24 h with cortisol at 250 μM. At the end of the treatments, mRNA was extracted and the cDNA levels of BDNF (a), SIRT-1 (b), CREB (c) and PRG-1 (d) were quantified by real-time PCR analysis. Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentage of untreated cells set to 1 (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni's post-test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs control; ### $p<0.001$ vs cortisol.

3.11. Effect of the nutraceutical combination treatment on inflammatory molecules expression

During aging, the CNS inflammation process increases. In particular, COX-2 levels increase is generally closely related to ROS formation [55]. These observations lead to examining whether the nutraceutical combination reduces in turn COX-2 levels. In the absence of cortisol, [1'] concentrations significantly reduced COX-2 levels, proving to have an effect even on basal levels of the enzyme. As expected, cortisol induced an increase in COX-2, but the mix was able to restore COX-2 physiological levels (Figure 10a), demonstrating that it possesses a basic anti-inflammatory capacity and an ability to prevent an increase in inflammation induced by oxidative stress mechanism. To deepen nutraceutical combination anti-inflammatory function, IL-6 and IL-8 were also quantified. Both interleukins participate in neurogenesis processes and their expression is affected in several of the main brain diseases [56, 57]. As Figures 10b,c show, in the absence of cortisol, nutraceutical combination did not cause any change in IL-6 or IL-8 levels, with respect to control. In contrast, the increase of interleukin, induced by cortisol, was prevented by the mix at both concentrations, with a greater action at the highest concentration. These data demonstrate that the mix is able to reduce oxidative stress-induced inflammation.

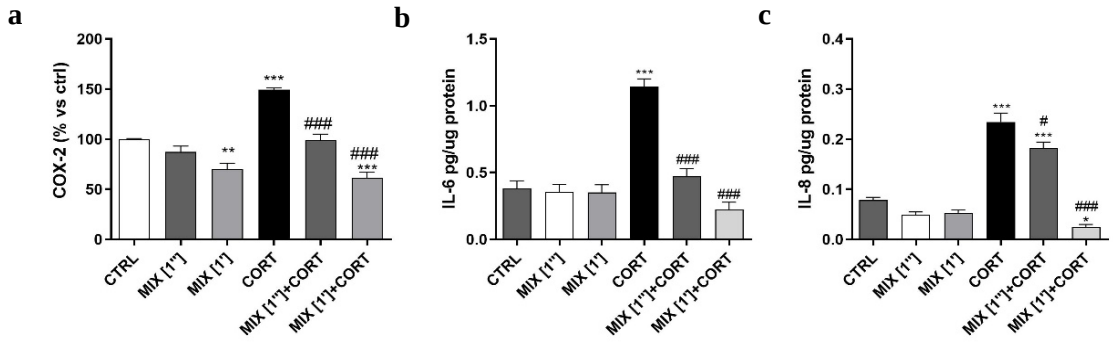

Figure 10. Evaluation of inflammatory signals. Following differentiation into cholinergic neurons, cells were treated with nutraceutical mix [1''] and nutraceutical mix [1'] for 24 h and for an additional 24 h with cortisol at 250 µM. COX-2 (a) and interleukins (b,c) levels were assessed by specific enzyme immunoassay. The data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni's post-test: ** $p<0.01$, *** $p<0.001$ vs control; $^{\#}$ $p<0.05$, $^{\#\#\#}$ $p<0.001$ vs cortisol.

4. Discussion

In the present study, neuroprotective properties of a nutraceutical combination were investigated in a model of cholinergic neurons. Specifically, the fix ratio of active compounds was demonstrated to have a protective role against mitochondrial dysfunction, inflammation and DNA damage. Moreover, challenging neuronal cells with this mix enhanced membrane fluidity and modulated overall gene transcription.

The effects of a specific ratio of omega-3 fatty acid, folic acid, vitamin E and magnesium on membrane and cellular well-being was verified human neuroblastoma cell line differentiated into cholinergic neurons. The cholinergic system is involved in learning, memory and age-related dementia [58] and represents a valuable cellular model for exploring the nootropic properties of compounds. For this purpose, cells were challenged with cortisol for 24 h in order to mimic physiological brain stress that can be related to aging [28]. In the present study, it was selected from a mix of several active nutraceuticals, known in literature to exert individually an activity in the physiology of the central nervous system, to test the possibility of a complementary and/or synergistic action. The fixed ratio of active ingredients present in the nutraceutical combination was initially chosen as they did not reduce cell viability by more than 20%.

First, to investigate the action of cortisol, an MTS and an apoptosis assay were performed. Consistent with our data, a cortisol treatment induced a decrease in HT22 cells viability and an increase in apoptosis [28], comparable to NaN_3 action in PC12 cells [39]. The mix significantly prevented cortisol- induced apoptosis. Consistently, EPA and DHA have been demonstrated to prevent cortisol-induced reduction in proliferation and increase in apoptosis in human hippocampal progenitor cell line [59]; at the same time, folic acid decreases astrocyte apoptosis by preventing oxidative stress-induced telomere attrition [60].

Assuming that high levels of cortisol regulate the expression of the ATP synthase genes at the mitochondrial level by reducing its expression [41], we challenged cholinergic neurons with cortisol and examined the ATP and ADP levels by calculating the ATP/ADP ratio. The results showed that nutraceutical combination significantly prevented the cortisol-mediated reduction of ATP and decrease in cell viability, at [1’] and [1’’] concentrations. Notably, these effects were mostly observed when the nutraceutical mix was administered following cortisol, thus evidencing the ability of the mix in restoring cortisol-induced energy imbalance and reverting its cytotoxic effects. Moreover, the effects displayed by the nutraceutical mix on ATP/ADP content were significantly higher with respect to those elicited by the single component, i.e., EPA/DHA, folic acid, vitamin E or magnesium, thus suggesting that additive/synergic effects may occur between the different nutraceutical components. Accordingly, literature reports similar synergistic cytoprotective or preventive effects of magnesium and Vitamin E [61], folic acid and vitamin B12 [62]. Among these papers, of particular relevance is the recent positive interaction of omega-3 fatty acid and vitamin E supplementation in improving working memory in older adults [63].

It is not clear why the mix [1 '] greatly increased the ATP/ADP ratio after the hydrocortisone application. These interesting results lead to hypothesize that, under physiological conditions, the supplement should not have particular effects on increasing ATP production, because the cellular energy request has been already satisfied. When the cell is in a state of prolonged stress, it is probable that the higher concentration of the nutraceutical combination has a greater capacity (compared to the concentration [1 "]) to restore the cellular basal conditions, due to an activation of several targets by the nutraceuticals. Consistent with our data, it has been reported that omega-3 supplementation delays age-related mitochondrial dysfunction in the brains of young and elderly mice [64]. In addition, by increasing the PGC1α and SIRT1 gene expressions, co-treatment with omega-3 and vitamin E has beneficial effects in patients with coronary artery disease, leading to an increase in ATP level [65]. On the other hand, magnesium stimulates the activity of several mitochondrial enzymes, and it is responsible for transporting ATP from mitochondria to the cytosol, which is mediated by an ATP-Mg / Pi carrier [11]; in particular, treatment of LmnaG609G/+ mice with dietary magnesium increased H+-coupled mitochondrial NADPH and ATP synthesis [66].

Following reduction of ATP levels, a serine/threonine kinase is activated [43], triggering autophagy processes [44]. In the absence of cortisol, nutraceutical combination treatment was able to reduce autophagy markers; similarly, following stimulus with cortisol, nutraceutical ingredients were able to significantly decrease the autophagy process at both concentrations. A considerable reduction was evident at [1’] concentration probably because higher mix concentrations favored the restoration of a cellular well-being environment, thus blocking autophagy. The physiological role and limits of autophagy are still debated in the current literature. This process is essential for maintaining proper cellular function and its dysfunction is associated with cancer, neurodegeneration, and aging [67]. Several works have demonstrated that mTOR inhibition by omega 3 induces autophagy processes, thus protecting cells from oxidative insults [68]. Conversely, other papers have demonstrated that antioxidant agents, including vitamin E, reduce autophagy markers in male Sprague-Dawley rats, relieving diabetic nephropathy [69], and that folic acid contrasts autophagosome formation in fatty liver disease rats [70]. In our hands, the presence of vitamin E and folic acid in the tested supplement, indeed composed by five-nutraceuticals, probably activates intracellular mechanisms that overcome omega 3-associated pathways, finally reducing the autophagic processes in our cellular model. In conclusion, we might speculate that the reduction of the autophagic process, in the absence of cortisol, could represent a beneficial mechanism. In conditions of oxidative stress, triggered by cortisol, nutraceutical combination exerted cytoprotective effects by inducing cellular autophagy.

During aging, prolonged exposure to high levels of glucocorticoids [5] in combination with low antioxidant defenses [6], can induce reactive oxygen species leading to oxidative damage in the CNS [38]. Challenging cholinergic neurons with nutraceutical combination significantly prevented the cortisol-induced ROS levels and DNA damage, both at [1’’] and [1’] concentrations. Notably, as evidenced for the ATP/ADP ratio, these effects were mostly maintained when the nutraceutical mix was administered following cortisol, thus evidencing the ability of the mix in restoring cortisol-induced imbalance in ROS production. Consistent with our data, omega-3 suppressed ROS formation in cultured neurons and neuronal progenitor cells of PPT1-free mice [71]; vitamin E protected cortical neurons against oxidative stress [72] and folic acid stabilized DNA, reducing damage [9]. Simultaneously, magnesium suppresses ROS production in various tissues, leading to the formation of magnesium–oxygen species [11]. Moreover, the effects displayed by the nutraceutical mix on ROS production were significantly higher with respect to those elicited by the single component, i.e., EPA/DHA, folic acid, vitamin E or magnesium. Similarly, Magnesium and Vitamin E co-supplementation have demonstrated a positive interaction on biomarkers of inflammation and oxidative stress in women with Polycystic Ovary Syndrome [61] and vitamin E has been proven to increase the biological effects of omega-3 fatty acids on redox balance in naturally aged rats [73].

In conditions of acute stress, the cell antioxidant mechanisms were responsible for restoring cellular well-being. One of the most important pathways is the Keap1/NRF2/ARE complex; under quiescent conditions, Nrf2 is anchored in the cytoplasm through binding to Keap1, which, in turn, facilitates the ubiquitination and subsequent proteolysis of Nrf2. Under stressful conditions, Nrf2 detaches from Keap1, translocates to the nucleus and activates a specific promoter sequence [74]. In contrast, in conditions of chronic stress, the Nrf2 pathway fails due to its degradation, depriving the cell of important antioxidant defenses [47]. Consistent with the latter evidence, challenging cholinergic neurons with cortisol for 24 h, induced a decrease in Nrf2 and Keap1 levels. Interestingly, the nutraceutical combination was able to prevent the cortisol-associated decrease in Nfr2 and Keap1 levels. These data are of particular relevance considering that Nfr2-Keap1 signaling has been linked to protective mechanisms against oxidative stress and proteotoxicity in cells, including neurons [75]. Similarly, omega-3 has demonstrated to improve the antioxidative defense in rat astrocytes and peritoneal macrophages via an Nrf2-dependent mechanism [12].

Brain aging is characterized by gene expression dysregulation of factors that influence neuronal plasticity and metabolism [1]. For this reason, we analyzed the expression of the CREB, BDNF, SIRT-1 and PRG1 genes [52, 51, 53, 54] in the SHSY-5Y cell line differentiated to cholinergic neurons and in the H9-derived NSC, in order to understand if the nutraceutical combination was able to regulate gene transcripts. These results demonstrated that the mix increases gene transcriptions of the mentioned transcripts, at [1’’] or [1’] concentrations and were equally able to prevent the reduction in cortisol-induced gene expression. Consistent with our data, omega-3 normalizes BDNF and CREB levels after traumatic brain injury in rats [76] and vitamin E enhances BDNF-TrkB-CREB signaling pathway in rats with cognitive impairment [77]. At the same time, omega-3 and vitamin E increase gene expression of SIRT1 and improve the response to oxidative stress in patients with coronary artery disease [65]. To date, no data in literature explored omega-3 or vitamin E effect on PRG1 expression. The lack of data on the action of folic acid on the expression of genes such as BDNF, CREB and SIRT1 could lie in the fact that its function is to prevent the accumulation of DNA damage in neurons caused by oxidative stress [78].

As a last evaluation step, the anti-inflammatory effects were tested in cholinergic neurons subjected to oxidative stress with cortisol. It is known that, during aging, CNS inflammation increases [5], therefore, COX-2 levels were evaluated as a value parameter linked to the presence of free radicals [79]. Challenging cells with the nutraceutical combination prevented cortisol-induced increase in inflammation. Consistent with our data, omega-3 is reported to show anti-inflammatory properties in rats with global ischemia [80], vitamin E inhibits COX-2 activity in human adenocarcinoma cells [81] and folic acid inhibits the proinflammatory action of COX-2 in rats given nicotine [82].

To deepen nutraceutical combination anti-inflammatory function, IL-6 and IL-8 were also quantified. Although neuronal cells are not largely deputed for the release of cytokines and pro-inflammatory molecules, literature reports that cytokines are proteins secreted in the central nervous system by microglia, astrocytes and

infiltrating peripheral immune cells and neurons too, under physiological and pathological conditions [83]. In the brain, cytokines are constitutively expressed in various brain regions by activated glial and neuronal cells [84, 34] and are involved in several normal and pathological processes including neuronal development, modulation of neurotransmitter metabolism and synaptic plasticity.

Challenging cells with the nutraceutical mix prevented cortisol-induced increase in IL-6 and IL-8 levels, with greater effectiveness at [1'] concentration. Consistent with our data, omega-3 were able to reduce inflammatory interleukins in a model of endothelial cells [85].

5. Conclusions

In human cholinergic neuronal-like cells, the nutraceutical combination was proven to: (i) prevent and revert the oxidative stress, energy imbalance and viability reduction induced by cortisol; (ii) prevent cortisol-induced DNA damage and disbalance in autophagy mechanism; (iii) increase membrane fluidity and transcription of genes related to cellular well-being; (iv) exert an anti-inflammatory effect by preventing COX2, IL-6 and IL-8 accumulation. Overall, these results confirm the complementary beneficial effects of simultaneous administration of omega-3, vitamin E, folic acid, and magnesium suggesting this nutraceutical combination as promising agent to preserve cholinergic neurons. In this sense, a recent report suggests the benefits of a combination of magnesium, folic acid, omega-3 fatty acids and vitamin E as a food supplement to complement brain functioning [86].

Author Contributions: E.Z. carried out biological experiments, analyzed the data and wrote the manuscript; S.D. was involved in scientific supervision and manuscript revision; L.C. and E.C. performed experiments and analyzed the data; C.M. was involved in funding acquisition and project supervision; M.V. was involved in scientific contribution and manuscript revision; G.M. was involved in project management, scientific contribution, and manuscript revision; L.D. was involved in scientific supervision, L.R. was involved in project supervision. All authors have read and agree to the published version of the manuscript.

Funding: This research was funded by Angelini Pharma S.P.A.

Declarations of interest: none.

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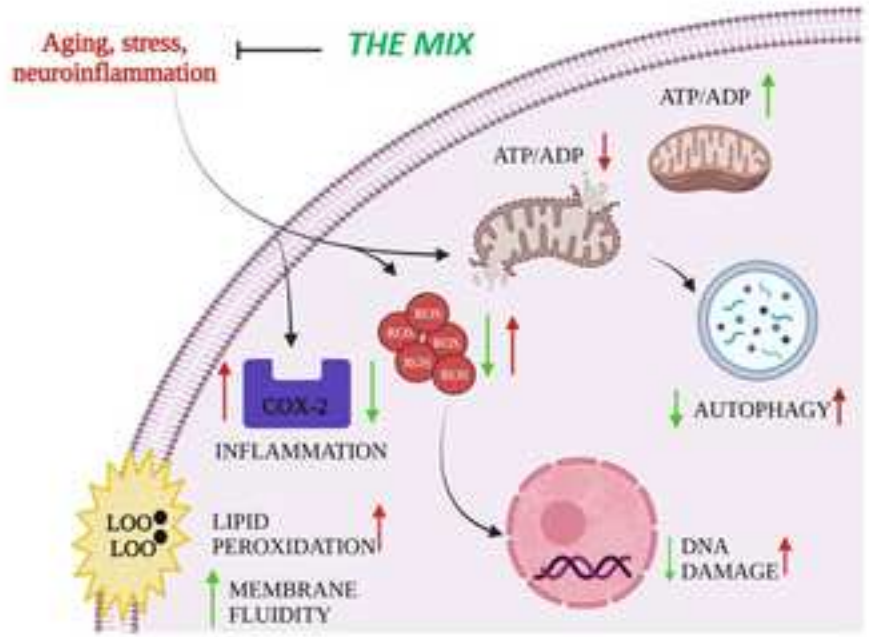
Graphical Abstract



A Specific Combination of Nutraceutical Ingredients exerts cytoprotective effects in human cholinergic neurons.

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Abstract
*Backgroud***:** Brain aging is associated with an excessive reactive oxygen species (ROS) formation that causes cell injury through proteins oxidation and DNA damage. These changes have been identified as contributing factors in age-related memory decline. In this sense, treatments able to protect central nervous system (CNS) from oxidative stress and to sustain membrane plasticity, may represent new candidates to counter the development of aging effects. Several studies have indicated vitamin E, folic acid, magnesium and omega-3 as nutraceuticals protecting CNS from oxidative stress.
Methods: A specific association of these active nutrients was tested in human cholinergic neurons, chosen as a cellular model related to learning and memory processes. Cortisol was used as an oxidative stress insult to explore the beneficial properties of the nutraceuticals.
Results: In summary, the specific ratio of active ingredients in the above selected food supplement prevented the decrease in ATP content and in cell viability exerted by cortisol. At the same time, it prevented ROS formation, DNA damage, autophagy processes and decrease in the expression of cellular well-being genes induced by cell treatment with cortisol. The effects on ATP content, ROS formation and cellular viability were evidenced when the nutraceutical mix when administered following cortisol treatment, too. Notably, these peculiar evidences were significantly higher with respect to those elicited by the single components of the food supplement.
Conclusions: Overall, these results confirm the beneficial effects of the simultaneous administration of vitamin E, folic acid, magnesium and omega-3.

Keywords: brain aging; vitamin E; folic acid; magnesium; omega-3; neuroprotection.

Abbreviations: 7-AAD, amino-actinomycin D; BDNF, brain-derived neurotrophic factor; bFGF basic fibroblast growth factor; ChAT, choline acetyltransferase; CNS, central nervous system; CORT, cortisol; CREB, cAMP response element-binding protein; DHA, docosahexaenoic acid; EGF, epidermal growth factor; EPA, eicosapentaenoic acid; FA, folic acid; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; H2DCFDA, fluorogenic dye 2′,7′-dichlorofluorescin diacetate; Keap1, kelch-like ECH-associated protein 1; LC3β, microtubule-associated protein light chain-3; Nrf2, nuclear factor-like 2; NSC, neural stem cells; PBS, phosphate buffered saline; PRG-1, plasticity-related gene 1; PUFA, polyunsaturated fatty acids; RA, retinoic acid; ROS, reactive oxygen species; SIRT-1, sirtuin.

1. Introduction

Brain aging has been associated with decline of biological functions, progressive memory loss, problems of coordination and difficulty in concentration [1]. During life, neuronal defense mechanisms decrease, leaving neurons exposed to free radical damage, protein accumulation and decreased neurogenesis [2, 3, 4]. Moreover, the reduction of the antioxidant defenses and the presence of high blood glucocorticoid levels [5], which characterize aging, could lead to an excessive reactive oxygen species (ROS) formation, causing inflammation through lipid peroxidation, glycosylation or oxidation of proteins and DNA damage [6]. Nutraceutical compounds can exert neuroprotective effects by regulating energy metabolism, neuro-oxidative stress, neuroinflammation and by improving neurogenesis. Herein, the nutraceutical combination used is a food supplement formulation containing a specific amount in a fixed ratio of active ingredients, including folic acid, vitamin E, magnesium and omega-3, which are essential to obtain the desired physiological balance [7]. Folic acid is an important vitamin for neuronal development [8] and for DNA stability. In fact, folate acts as a donor of methyl groups leading to the formation of S-adenosylmethionine which regulates DNA transcription and gene stability [9]. At the same time, vitamin E is a lipid-soluble antioxidant vitamin capable of blocking radical chain reactions and regulating signal transduction [10]. Another critical component for the maintenance of CNS functions is magnesium. Indeed, it is the second most important and abundant cation in cells, and it is essential for many physiological processes. Maintaining the appropriate magnesium concentrations is necessary for cell growth, proliferation, differentiation, energy metabolism and death [11]. Omega-3 are essential unsaturated fatty acids (n-3 PUFAs) and they are crucial components of neuronal cell membranes; among these, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are pivotal for antioxidant defenses [12] and to preserve the correct membrane cell fluidity [13]. Actually, the maintenance of a correct cell membrane plasticity allows to obtain a good cellular communication and to counteract memory and learning deficits related to the changes in neuronal and synaptic density [14, 15]. In this sense, maintaining adequate levels of these fatty acids leads to a reversion of age-related synaptic plasticity changes, a reduction of inflammation and brain atrophy [16]. In this context, treatments able to reduce inflammation, to increase neurotropism and to protect CNS from oxidative stress, may represent new candidates to counteract aging development and neuro-psychological alterations [17]. Currently, treatments composed of one or two ingredients, among folic acid, vitamin E, magnesium and omega-3 have been studied, but all together (as a mixture), at specific concentrations, have not been assessed yet. Although present in specific food sources not always easy to get, these nutraceutical compounds can be provided also through a healthy and balanced diet. In fact, n-3 PUFAs such as EPA and DHA are contained in fatty cold-water fishes (mackerel, sardine, herring, tuna, and salmon), fish oil, seafood, and krill oil [18, 19, 20]. The major dietary sources of vitamin E are vegetable oils (e.g., olive, coconut, sunflower, palm, soybean), whole grains, leafy vegetables, and nuts [21, 22, 23], while magnesium is taken frequently through dietary sources such as green leafy vegetables, cocoa, almonds, nuts, unground grains, whole seeds, legumes, and at lower concentrations through fruit, meat (chicken, pork), and fish [24, 25]. Finally, folic acid is the synthetic form of folates, used in supplements or fortified foods, while folates are provided naturally by eating green leafy vegetables, yeast, liver, eggs, fermented products (e.g., cheese, yogurt), legumes, and some fruits [26, 27]. In the present work, the active ingredients were combined according to a peculiar percentage reported in Libretto®/Primus® and tested in human cholinergic neuronal-like cells (i.e., differentiated SHSY-5Y cells) for their abilities to protect neurons against age-related cellular alterations [28].

For the purpose of the study, the SH-SY5Y culture system has been chosen, which is a convenient neuronal model. This model has the potential to elaborate human/primate-specific transcription networks and pathways related to human cognitive disorders [29], which has been already frequently used to explore neurodegeneration, oxidative stress, and psychological disorders [30]. In particular, recent literature has reported that SHSY-5Y cell differentiation with retinoic acid (RA) and brain-derived neurotrophic factor (BDNF), as in the present paper, induces the expression of acetylcholinesterase and choline acetyltransferase enzymatic activities and it has been proposed as a suitable model of cholinergic neurons to study cellular processes associated to aging and neurodegeneration [31].

2. Materials and Methods

2.1 Materials

cis-5,8,11,14,17-eicosapentaenoic acid (EPA), cis-4,7,10,13,16,19-docosahexaenoic acid (DHA), folic acid (FA), D-alpha-tocopherol polyethylene glycol (vitamin E), magnesium carbonate (Mg^{2+}) hydrocortisone for cortisol (CORT), retinoic acid (RA) and brain-derived neurotrophic factor (BDNF) were purchased from Sigma Aldrich. All other reagents were obtained from standard commercial sources. In this paper, the complete ingredients mix (Libretto®/Primus®, Angelini Pharma S.p.A) "nutraceutical combination" or as a "nutraceutical mix" and it was used in two set of dilutions: MIX [1'] refers to 8 μM EPA, 3.12 μM DHA, 4.5 nM folic acid, 52 nM vitamin E and 2.8 μM magnesium while MIX [1''] refers to 0.8 μM EPA, 312 nM DHA, 0.45 nM folic acid, 5.2 nM vitamin E and 280 nM magnesium. EPA, DHA and folic acid were diluted to different concentrations of stock solutions by 100% DMSO; otherwise, vitamin E and magnesium were diluted by water. All subsequent dilutions were carried out in water, therefore in the final composition of the nutraceutical, there are negligible amounts of each solvent.

2.2 Cell cultures differentiation and induction of stress

SH-SY5Y neuroblastoma cell line was cultured in a complete medium consisting of DMEM, 10% fetal bovine serum (FBS), and 2 mM L-glutamine, at 37 °C in 5% CO_2. For cholinergic neuronal differentiation, SH-SY5Y were cultured in DMEM reducing FBS concentration to 2% and supplementing with 10 μM RA for 7 days. This treatment was replaced every 2 days. On the fourth day of differentiation 50 ng/mL of BDNF were added [31]. To set up the stress model, an MTS assay was performed and hydrocortisone (water soluble, H0396, Sigma Aldrich, Milan, Italy) at 250 μM for 24 h was used on human neurons. H9-derived Neural Stem Cells (NSCs) were purchased from GIBCO (Life Technologies, Milan, Italy). They were cultured in complete medium consisting of KnockOut™D-MEM/F-12 with StemPro® Neural Supplement, 20 ng/ml of basic fibroblast growth factor (bFGF, Life Technologies, Milan, Italy), 20 ng/ml of epidermal growth factor (EGF, Life Technologies, Milan, Italy), and 2 mM L-glutamine at 37 °C in 5% CO_2.

2.3 Immunohistochemistry

Cell differentiation was evaluated using immunofluorescence. Cells were washed with phosphate buffered saline (PBS), fixed with paraformaldehyde (4% v/v) for 20 min at room temperature (RT), and permeabilized with 0.1% Triton X-100-supplemented PBS. Nonspecific binding was blocked with 2.5% BSA for 1 h at RT. After PBS washes, cells were incubated with anti-ChAT (abcam, ab178850) antibody, overnight at 4°C. Subsequently, a secondary antibody anti-rabbit Alexa Fluor® (Invitrogen, 1:500) was used, for 45 min at RT [32]. Ten microscopic fields (× 60 magnification) were selected randomly from each of three independent experiments (n= 3). The images were obtained with a Zeiss fluorescence microscope equipped with ApoTome technology (Zeiss Microscopy, Jena, Germany) and analyzed with ImageJ software. To obtain a quantitative indication of differentiated cells, Alexa Fluor®-positive cells were counted, and the percentage of ChAT-positive cells was calculated by using DAPI labelling to obtain the total cell number. Subsequently, with the ImageJ program the relative fluorescence was calculated in control and differentiated cells. DAPI labelling was used to normalize fluorescence intensity to the same number of cells. The data were expressed as fold change of fluorescence intensity vs control cells.

2.4 Apoptosis Assessment

SH-SY5Y neuroblastoma cell line was seeded in 6-multiwell plates (10000 cells/ well or 5.000 cell/ml). After differentiation into cholinergic neurons (1 week), cells were treated with nutraceutical combination at [1'] and [1''] concentrations for 24 h and subsequently with hydrocortisone or sodium azide (NaN_3) for an additional 24 h. After treatment, early and late apoptotic SH-SY5Y were estimated by Muse Apo Assays (Merck-

Millipore) as previously reported [33]. Apoptotic and dead cells were distinguished using the annexin V conjugated with fluorescein isothiocyanate (FITC) and amino-actinomycin D (7-AAD) [33].

2.5 ATP and ADP assay

SH-SY5Y neuroblastoma cell line was seeded in 96-multiwell plates (5000 cells/ well or 50.000 cells/ml). After differentiation into cholinergic neurons (1 week), cells were treated with 8 μM EPA, 3.12 μM DHA, 4.5 nM folic acid, 52 nM vitamin E or 2.8 μM magnesium, or the nutraceutical combination at [1''] and [1'] concentrations for 24 h. Following incubation time, cells were incubated with hydrocortisone for an additional 24 h. In contrast, in "post-treatment experiments", cells were incubated with hydrocortisone and then challenged with the nutraceutical combination for 24 h. At the end, the amount of ATP and ADP were estimated using a bioluminescence assay kit, according to the manufacturer's instruction (ADP/ATP Ratio Assay Kit, Abcam, Milan, Italy) [33]. The luminescence data were reported as the ATP/ADP ratio vs control cells set to 100%.

2.6 Cell proliferation assay

SH-SY5Y neuroblastoma cell line was seeded in 96-multiwell plates (5000 cells/ well or 50.000 cells /ml) and differentiated into cholinergic neurons, as described above. Moreover, H9-derived NSCs were used in parallel experiments to investigate the effects of the supplement on stem cells viability. SH-SY5Y neuroblastoma cell line was treated with 8 μM EPA, 3.12 μM DHA, 4.5 nM folic acid, 52 nM vitamin E or 2.8 μM magnesium, or the nutraceutical combination at [1''] and [1'] concentrations for 24 h. Following incubation time, cells were incubated with hydrocortisone for an additional 24 h. In contrast, in "post-treatment experiments", cells were incubated with hydrocortisone and then challenged with the nutraceutical combination for 24 h. At the end of treatments, cell proliferation was determined using the MTS assay according to the manufacturer's instruction. Within an experiment, each condition was analyzed in triplicate, and each experiment was performed at least three times. The results were calculated by subtracting the mean background from the values obtained from each test condition. Final data were reported as the fold change from control cells set to 100% [34].

2.7 Western blot analysis

SH-SY5Y neuroblastoma cell line was seeded in 6-multiwell plates (10000 cells/well or 5.000 cells/ml). After differentiation into cholinergic neurons (1 week), cells were treated with nutraceutical combination at [1''] and [1'] concentrations for 24 h. Following incubation time, cells were exposed to 250 μM of hydrocortisone for an additional 24 h. At the end of the treatment period, cells were collected and then were lysed for 2 hours at 4 °C in RIPA buffer (9.1 mM NaH_2PO_4, 1.7 mM Na_2HPO_4, 150 mM NaCl, pH 7.4, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1 % SDS, and a protease-inhibitor cocktail). Equal quantities of the cell extracts (50 μg of protein) were diluted in Laemmli solution, resolved using SDS-PAGE (8.5%), transferred to PVDF membranes, and probed overnight at 4 °C using an antibody anti-choline acetyltransferase (ChAT, ab178850 abcam, 1:200) anti-microtubule-associated protein light chain-3 (LC3β, sc-28266 Santa Cruz Biotechnology, 1:100) [35] or anti-oxidative proteins nuclear factor-like 2 (Nrf2, ab137550 abcam, 1:200) or kelch-like ECH-associated protein 1 (Keap1, AV38981 Sigma-Aldrich, 1:200). The primary antibody was detected using the appropriate peroxidase-conjugated secondary antibody, which was then detected using a chemiluminescent substrate (ECL, Perkin Elmer). Densitometric analysis of the immunoreactive bands was performed using Image Lab Software. The densitometric analysis was performed taking into consideration both bands relating to the two different forms of LC3β. The data are reported as the fold change from control cells set to 100%. All western blots were performed with the ChemiDoc XRS+Gel Imaging System (Biorad, Milan, Italy), which includes gel activation and acquisition of the total proteins imprinted in the PVDF. This "stain-free protein normalization" allows the normalization of bands to total protein in blots, thus eliminating the need for housekeeping proteins.

2.9 ROS assay

SH-SY5Y neuroblastoma cell line was seeded in 96-multiwell black plates (5000 cells/ well). After differentiation into cholinergic neurons (1 week), cells were treated with 8 μM EPA, 3.12 μM DHA, 4.5 nM folic acid, 52 nM vitamin E or 2.8 μM magnesium, or the nutraceutical combination at [1''] and [1'] concentrations for 24 h. Following incubation time, cells were exposed to 250 μM of hydrocortisone for an additional 24 h. In contrast, in "post-treatment experiments", cells were incubated with hydrocortisone and then challenged with the nutraceutical combination for 24 h. ROS activity was determined using the fluorogenic dye 2′,7′-dichlorofluorescin diacetate (H2DCFDA, Molecular Probes, Invitrogen). Following incubation with treatments, cells were washed and a solution of PBS + glucose 10 mM with 50 μM of H2DCFDA was added in the dark at 37 °C for 30 minutes. The fluorescence intensity (excitation 485 nm and emission 520 nm) was normalized based on the number of cells stained with crystal violet [33]. Data were reported as the fold change from control cells set to 100%.

2.10 Quantification of DNA damage

SH-SY5Y neuroblastoma cell line was seeded in 96-multiwell plates (5000 cells/ well). After differentiation into cholinergic neurons (1 week), cells were treated with nutraceutical combination at [1''] and [1'] concentrations for 24 h; at the end of treatments, cells were incubated with 250 μM of hydrocortisone for an additional 24 h. Following, cells were lysed, and equal amounts of protein were incubated in precoated wells. The DNA damage was estimated using an IR assay kit (Human H2A.X phospho S139 ELISA Kit, Abcam, Milano, Italy) according to manufacturer's instructions [33]. The data are reported as fold change over control cells (100%).

2.11 Evaluation of membrane fluidity

SH-SY5Y neuroblastoma cell line was seeded in 96-multiwell plates (5000 cells/ well). After differentiation into cholinergic neurons (1 week), cells were treated with nutraceutical combination at [1''] and [1'] concentrations for 24 h. Following incubation time, cells were exposed to 250 μM of hydrocortisone for an additional 24 h. Membrane fluidity was measured by flow cytometry using a fluorescent labelling method according to the manufacturer's protocol (Membrane Fluidity kit, Abcam, Milan, Italy). The EnSight™ multimode plate reader, measuring the excitation at 350 nm and the emission at 470 nm (excimer) and 370 nm (monomer), was used for analyzing the fluorescence intensity of labeled cells. Quantitative monitoring of the membrane fluidity was obtained by measuring the ratio of monomer to excimer fluorescence [36]. The data are reported as fold change over control cells (100%).

2.12 RNA extraction and real-time PCR analysis

SH-SY5Y neuroblastoma cell line was seeded in 6-multiwell plates (10.000 cells/ well or 5.000 cells/ml) and differentiated into cholinergic neurons. In parallel experiments, H9-derived NSCs were used to investigate the effects of the supplement on stem cells. Both cell lines were treated with nutraceutical combination at [1''] and [1'] concentrations for 24 h; after the incubation time, cells were exposed to 250 μM of hydrocortisone for an additional 24 h. At the end of treatments, cells were collected, and total RNA was extracted using Rneasy® Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The purity of the RNA samples was determined by measuring the absorbance at 260:280 nm and cDNA synthesis was performed with 500 ng of RNA using i-Script cDNA synthesis kit (BioRad, Hercules, USA). Primers used for RT-PCR were designed in intron/exon boundaries to make sure that products did not include genomic DNA [34]. RT-PCR reactions consisted of 10 μL Fluocycle® II SYBR® (Euroclone, Milan, Italy), 0.6 μL of both 10 μM forward and reverse primers, 5 μL cDNA, and 3.8 μL of H_2O. All reactions were performed for 40 cycles using the following temperature profiles: 98 °C for 30 s (initial denaturation); T °C (see Table 1) for 30 s (annealing); and 72 °C for 3 s (extension) [36]. β-actin was used as the housekeeping gene. PCR specificity was determined

by both the melting curve analysis and gel electrophoresis. The data were reported as fold change over control cells set to 1.

Gene	Primer nucleotide sequences	Product size (base pairs)	Annealing temperature
BDNF	FOR: 5'-TACATTTGTATGTTGTGAAGATGTTT-3' REV: 5'-CCTCTTTTCAGAAAAATTCAGGA-3'	131	56°
SIRT1	FOR: 5'-CCTGGACAATTCCAGCCATC-3' REV: 5'-TTCATGATAGCAAGCGGTTCAT-3'	272	66°
CREB	FOR: 5'-AAGCTGAAAGTCAACAAATGACA-3' REV: 5'-CCTCTTTTCAGAAAAATTCAGGA-3'	240	52°
PRG-1	FOR: 5'-CCCGCTCAGGGAATAGCTG-3' REV: 5'-GCTAACCACCGATGATGCCA-3'	156	54°
B-actin	FOR: 5'-GCACTCTTCCAGCCTTCCTTCC-3' REV: 5'-GAGCCGCCGATCCACACG-3'	54	55°

Table 1. Nucleotide sequences and annealing temperature of the primers utilized in PCR.

2.13 COX-2 assay

SH-SY5Y neuroblastoma cell line was seeded in 96-multiwell plates (5000 cells/ well). After differentiation into cholinergic neurons (1 week), cells were treated with nutraceutical combination at [1''] and [1'] concentrations for 24 h. Following incubation time, cells were exposed to 250 µM of hydrocortisone for an additional 24 h. The amount of COX-2 in supernatant was estimated using an enzyme-linked immunosorbent assay (Human Cox-2 ELISA kit, Sigma Aldrich) according to manufacturer's instructions. Data were reported as a fold change over control cells set to 100%.

2.14 IL-6 and IL-8 assay

SH-SY5Y neuroblastoma cell line was seeded in 96-multiwell plates (5000 cells/ well). After differentiation into cholinergic neurons (1 week), cells were treated with nutraceutical combination at [1''] and [1'] concentrations for 24 h. Following incubation time, cells were exposed to 250 µM of hydrocortisone for an additional 24 h. The interleukins content in the supernatant was measured using enzyme-linked immunosorbent assay (ELISA) kits (Cloud-Clone Corp., Katy, Texas, United States: SEA079Hu for IL-6, SEA080Hu for IL-8) following the manufacturers' instructions. Briefly, 100 µL of supernatant was added into the appropriate wells and incubated for 1 hour at 37 °C. After incubation time, 100 µL of primary antibody were added for 1 hour at 37 °C. After extensive washes, 100 µL of secondary antibody were incubated for 30 minutes at 37 °C and then, the substrate solution was added to each well, leaving the color to develop for 10-20 min at 37 °C. Absorbance was measured at 450 nm and a standard curve was obtained with standards kit.

2.15 Statistical Analysis

Data analysis was performed using one-way analysis of variance (ANOVA) with "Bonferroni's Multiple Comparison Test". $p<0.05$ was considered statistically significant.

3. Results

3.1. Differentiation of SHSY-5Y human neuroblastoma cell line into cholinergic neurons

Aging is characterized by loss of some cholinergic system functions [37]. To investigate the effect of nutraceutical combination, human neuroblastoma cell line SHSY-5Y was differentiated into a cholinergic neuronal system with retinoic acid (RA) and brain-derived neurotrophic factor (BDNF) for seven days [31]. To confirm SH-SY5Y differentiation in cells with characteristics of cholinergic neurons, the expression of choline acetyltransferase (ChAT) enzyme was evaluated by Western blot analysis. The induction of differentiation led to an increase in the expression of protein involved in the production of acetylcholine, demonstrating the acquisition of typical characteristics of cholinergic neurons (Supplementary Figure 1 a,b). To confirm the results obtained with western blot analysis, an immunostaining of ChAT was performed (Supplementary Figure 1 c,d). In control undifferentiated cells, 25±3% of total cells were positive to ChAT, confirming the basal expression of the selected protein in these neuronal-like cells [31]. Following cell treatment, 80±6% of cells expressed ChAT, thus demonstrating that RA plus BDNF significantly induced SHSY-5Y differentiation into cholinergic neurons. Quantification of the relative fluorescence intensity, normalized to the same cell number, showed that differentiation induced a 1.76-fold increase in fluorescence intensity (Supplementary Figure 1d).

3.2. Effect of cortisol on cell viability and evaluation of cortisol induced apoptosis

Assuming that an increase in glucocorticoid levels has been observed with aging [5] and that chronic and non-physiological levels of cortisol induce an increase in oxidative stress leading to the formation of reactive oxygen species (ROS) [38], a cortisol stress model [28] was developed to evaluate and to corroborate nutraceutical mix neuroprotective effects. For this purpose, cells were treated with two different concentrations (250 µM and 500 µM) of cortisol (CORT) for different times (4,8,16,24 h) and then, cell viability was assessed (Figure 1a). The concentration of 500 µM caused a reduction in cell viability of about 55%, almost mimicking acute stress. The lowest concentration (250 µM) produced a less marked reduction of cell viability, also at longer incubation times. Based on the obtained results, and in order to simulate a daily chronic physiological stress, this lower concentration for the longer time (24 h) was chosen.
In order to study the effect of cortisol on cellular conditions, apoptotic staining was performed. In parallel, sodium azide, an inhibitor of the fourth mitochondrial complex with apoptotic effect, was used to compare the behavior of cortisol [39, 40]. As Figure 1 (panel b) shown, the trend of cortisol (24.57% in early apoptosis and 12.36% in late apoptosis) appeared very similar to sodium azide (23.57% in early apoptosis and 11.23% in late apoptosis), demonstrating to have analogous effects. In supplementary materials (Supplementary Figure 2 a,b), we demonstrated that both mixes were able to protect cells from apoptosis damage cortisol-induced.

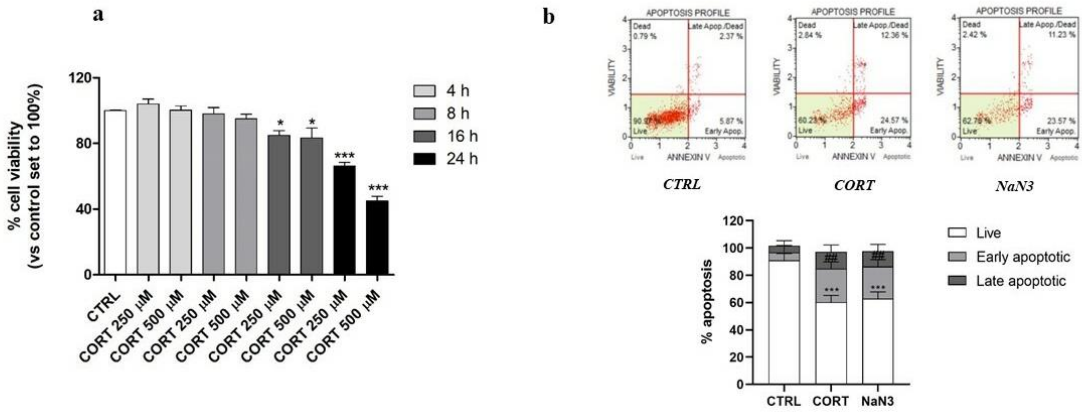

Figure 1. Stress model with cortisol. (a) The SH-SY5Y cell line was differentiated into cholinergic neurons and after they were treated with cortisol at concentrations of 250 µM and 500 µM for 4, 8, 16, and 24 h. Following incubation, cellular viability was measured by MTS assay. Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni's post-test: * $p<0.05$, *** $p<0.001$. (b) The SH-SY5Y cell line was differentiated into cholinergic neurons and after, they were treated with cortisol at concentration of 250 µM or with sodium azide at concentration of 3 mM for 24 h. Following incubation, cellular apoptosis was measured by Muse Apo Assays (Merck-Millipore) as previously reported [33]. The data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni's post-test: *** $p<0.001$ and $^{\#\#}$ $p<0.01$ versus control of live cells and early apoptotic cells, respectively.

3.3. Assessment of suitable concentrations of the nutraceutical combination

In order to establish the safe and non-toxic concentrations of nutraceutical mix, cellular viability was tested for each nutraceutical compound. At the same time, particular concentrations were chosen to maintain an exact and specific proportion between compounds within the mix (Figure 2). As shown in Figure 2 the mix containing 8 µM EPA, 3.12 µM DHA, 4.5 nM folic acid, 52 nM vitamin E and 2.8 µM magnesium, here indicated as mix [1'], was initially chosen as single ingredients that did not reduce cell viability by more than 20%. Subsequently, the mix was further diluted (ten-fold reduction) and used as final concentrations of active ingredients (0.8 µM EPA, 312 nM DHA, 0.45 nM folic acid, 5.2 nM vitamin E and 280 nM magnesium). Here, this second mix is indicated as mix [1''] and it was chosen to assess if the beneficial effects can be maintained even at lower concentrations too (Figure 2).

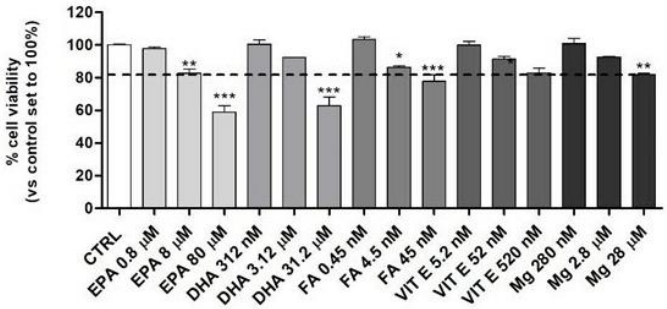

Figure 2. Assessment of mix concentrations. The SH-SY5Y cell line was differentiated into cholinergic neurons, and, after, they were treated with different dilution of active ingredients of nutraceutical combination. Then, two sets of concentrations were chosen, indicated as: mix [1''] and mix [1']. Following incubation, cellular viability was measured by MTS assay. Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni's post-test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs control.

3.4. Effect of the nutraceutical combination on ATP/ADP ratio and cell viability

In light of recent publications and insights on the action of glucocorticoid receptors at mitochondrial level [41], we considered appropriate to examine the cellular energy balance as an index of mitochondrial dysfunction and mitochondrial protective potential of nutraceutical mix. As depicted in Figure 3, in the absence of cortisol, the mix did not cause any significant change in ATP levels, both at [1''] and [1'] concentrations. As expected, the action of cortisol on glucocorticoid receptors resulted in energy damage; while, in contrast, nutraceutical mixes were able to prevent the cortisol-induced imbalance of ATP/ADP ratio, suggesting an involvement of supplements in restoring ATP levels (Figure 3a). Notably, similar results were obtained when the mix was administered following cortisol (Figure 3a), thus suggesting that the nutraceutical combination can rescue the metabolic alterations induced by cellular stress, too. The effects displayed by the nutraceutical mix on

ATP/ADP content were significantly higher with respect to those elicited by the single component, i.e., EPA/DHA, folic acid, vitamin E or magnesium (Supplementary Figure 3a).
Due to the ATP reduction, an assay of cell viability was assessed. In the absence of cortisol, the mix did not cause reduction in cell viability, neither at [1’’] and [1’] concentrations; as expected, challenging cells with cortisol significantly decreased cell viability but this reduction was prevented by challenging cells with both mix (Figure 3b). Notably, a significant rescue in cortisol-induced decrease in cellular viability was obtained when the mix was administered following cortisol (Figure 3b). The cytoprotective effects elicited by the nutraceutical combination did not significantly differ from those obtained by testing the single components alone, i.e., EPA/DHA, folic acid, vitamin E or magnesium (Supplementary Figure 3b).
In order to study the effect of nutraceutical mix on the viability of a stem cell line, H9-derived NSCs were chosen. The mix displayed similar effects in this cell line: specifically, in the absence of cortisol, it did not cause reduction in cell viability, either at [1 "] or [1 '] concentrations; as expected, challenging cells with cortisol significantly decreased NSC viability (Supplementary Figure 4). The decrease in NSC viability was prevented by nutraceutical mix (Supplementary Figure 4). These data evidenced a cytoprotective effect of this nutraceutical combination in cholinergic neurons and in neural stem cells.

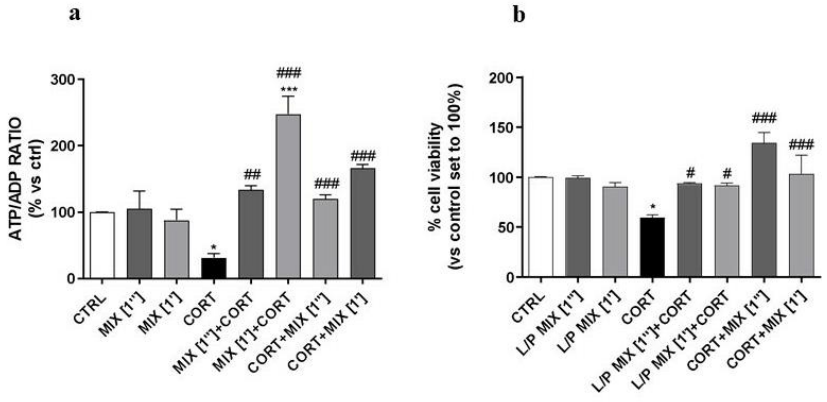

Figure 3. (a-b) Evaluation of the ATP/ADP ratio (energy balance) and cell viability. Following differentiation into cholinergic neurons, cells were treated with nutraceutical mix at [1’’] and [1’] concentrations, for 24 h and for an additional 24 h with cortisol at 250 μM (MIX[1’’]+CORT or MIX[1’]+CORT). In post-treatment experiments (CORT+MIX[1’’] or CORT+MIX[1’]), differentiated cells were challenged with 250 μM cortisol for 24 h, and then with the nutraceutical mix at [1’’] and [1’] for an additional 24 h. The ATP/ADP ratio was evaluated by specific fluorometric assay (a), and cellular viability was measured by MTS assay (b). Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni’s post-test: * $p<0.05$, *** $p<0.001$ vs control; # $p<0.05$; ## $p<0.01$, ### $p<0.001$ vs cortisol.

3.5. Effect of nutraceutical combination treatment on autophagy pathway

Autophagy is a mechanism that allows the degradation and recycling of cellular components. During this process, the damaged cytoplasmic constituents are isolated from the rest of the cell within a double-membrane vesicle known as autophagosome. The formation of the autophagosome occurs when the cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form the conjugated form LC3-phosphatidylethanolamine (LC3-II). At this stage, the autophagosome membrane fuses with that of a lysosome and the contents are degraded and recycled [42]. Since ATP reduction activated a serine/threonine kinase (AMPK) [43], triggering autophagy processes [44], the influence of the food supplements on autophagy phenomena was investigated. As reported in literature autophagic activity at the neuronal level is constitutively and physiologically higher than in other districts [45]. For this reason, in the absence of cortisol, both bands related to LC3β-I and LC3β-II protein were evident, validating the constitutive autophagic hypothesis of neuronal cells (Figure 4a). The increase in cortisol-induced autophagy was prevented by the following treatment with nutraceutical mix at both concentrations (Figure 4b). This signal may suggest a restoration of cellular balance and a consequent reduction of autophagy processes.

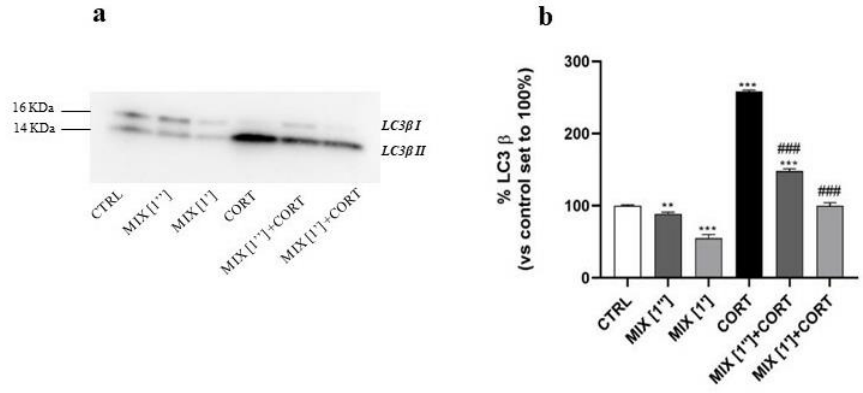

Figure 4. (a-b) Evaluation of autophagy induction: LC3β. Following differentiation into cholinergic neurons, cells were treated with nutraceutical mix [1'']and nutraceutical mix [1'] for 24 h and for an additional 24 h with cortisol at 250 μM. After incubation, cells were collected and lysed. Autophagic marker LC3 (I and II) levels were assessed by western blot analysis using the anti-LC3β antibody. A representative Western blotting is shown (a). Irrelevant parts of the gel image are deleted. Optical density was measured by Image Lab software (b). Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as percentage of untreated cells (control set to 100%). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni's post-test: ** $p<0.01$, *** $p<0.001$ vs control; $^{###}$ $p<0.001$ vs cortisol.

3.6. Effect of nutraceutical combination treatment on ROS levels

Prolonged exposure to stress determines an abnormal and continuous secretion of glucocorticoids, leading to oxidative damage in the CNS [38]. In the absence of cortisol, nutraceutical mix did not cause an increase in oxidative damage, at [1'']and [1'] concentrations. As expected, cortisol treatment significantly increased ROS levels (Figure 5). Challenging cells with the mix for 24 h before oxidant damage significantly prevented ROS formation at both sets of concentrations (Figure 5 MIX[1'']+CORT or MIX[1']+CORT). These data demonstrate that this nutraceutical combination protects against oxidative stress. Notably, similar results were obtained when the mix was administered following cortisol (Figure 5 CORT+MIX[1''] or CORT+MIX[1']), thus suggesting that the nutraceutical combination can rescue ROS imbalance induced by cellular stress, too.

The effects displayed by the nutraceutical mix on ROS production were significantly higher with respect to those elicited by the single component, i.e., EPA/DHA, folic acid, vitamin E or magnesium (Supplementary Figure 5).

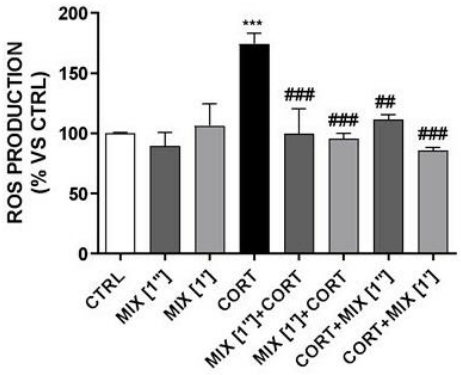

Figure 5. Evaluation of ROS levels. Following differentiation into cholinergic neurons, cells were treated with nutraceutical mix [1'’] and nutraceutical mix [1’] for 24 h and for an additional 24 h with cortisol at 250 µM (MIX[1'’]+CORT or MIX[1’]+CORT). In post-treatment experiments, differentiated cells were challenged with 250 µM cortisol for 24 h, and then with the nutraceutical mix at [1'’] and [1’] for an additional 24 h (CORT+MIX[1'’] or CORT+MIX[1’]). Oxidative stress levels were assessed by H2DCFDA fluorometric probe. Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni’s post-test: *** $p<0.001$ vs control; ## $p<0.01$, ### $p<0.001$ vs cortisol.

3.7. Effect of cortisol treatment on anti-oxidative proteins

Generally, the increase in oxidative stress determines an increase in cellular antioxidant defenses. Under acute stress conditions, Nrf2 dissociates from Keap1 and translocates into the nucleus where it may act as a transcription factor for antioxidant response genes [46]. In conditions of chronic stress, the Nrf2 pathway fails due to its degradation, depriving the cell of important antioxidant defenses [47].

To explore the effect of nutraceutical mix on antioxidant defenses, a western blot assay was performed to detect Nrf2 and Keap1 proteins. As depicted in Figure 6a,a’, cortisol treatment induced a significant decrease in Nrf2 proteins levels, respectively. In contrast, the mix, tested at both concentrations, demonstrated the ability to prevent Nfr2-decrease induced by cortisol.

These results demonstrate that cortisol mediates a reduction in the antioxidant activity of Nrf2-Keap1 complex, and that this nutraceutical combination is able to preserve the activity of Nrf2-Keap1 axis.

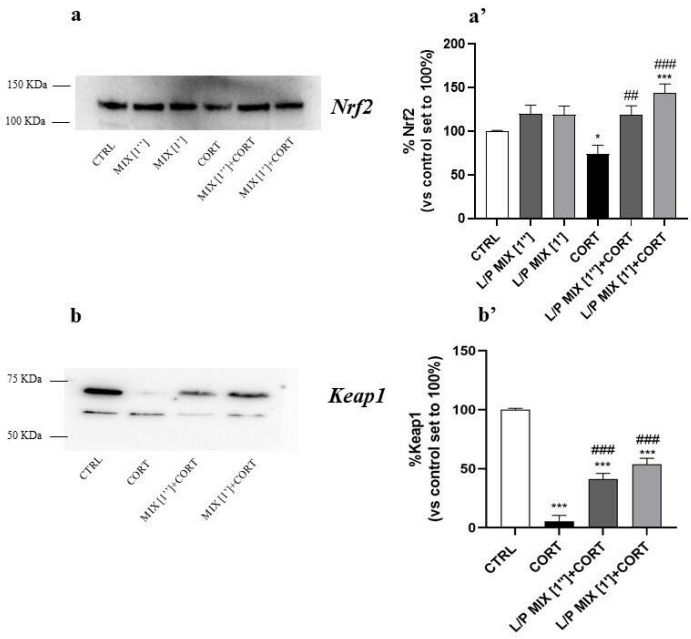

Figure 6. Evaluation of antioxidant protein levels. Following differentiation into cholinergic neurons, cells were treated with nutraceutical mix [1'’] and nutraceutical mix [1’] for 24 h and for an additional 24 h with cortisol at 250 µM. After incubation, cells were collected and lysed. Anti-oxidative proteins kelch-like ECH-associated protein 1 (Keap1) and nuclear factor-like 2 (Nrf2) levels were assessed by western blot analysis using the anti- Keap1 or anti- Nrf2 antibodies. A representative Western blotting is shown (a,b). Optical density was measured by Image Lab software (a’,b’). Data are the mean ± SEM of three different experiments and are reported as the percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni’s post-test: * $p<0.05$, *** $p<0.001$ vs control; $^{\#\#}$ $p<0.01$, $^{\#\#\#}$ $p<0.001$ vs cortisol.

3.8. Effect of the nutraceutical combination treatment on DNA damage

Prolonged stress leads to an increase in reactive oxygen species [38] which in turn are responsible for DNA damage [48]. Considering that this nutraceutical combination was able to reduce stress-induced oxidative damage, we next verified whether it could preserve human neurons from DNA damage. For this purpose, histone H2AX phosphorylation was evaluated as a marker of DNA damage [49]. As depicted in Figure 7, the mix was able to reduce histone H2AX phosphorylation at both sets of dilutions tested, demonstrating to have an intrinsic capacity in DNA protection. As expected, cortisol significantly increased histone H2AX phosphorylation; this increase was significantly contrasted with supplement mix at both sets of concentrations (Figure 7). Taken together, these results demonstrate that pre-treatment of human neurons with this nutraceutical combination reduces histone H2AX phosphorylation and prevents cortisol-induced DNA damage.

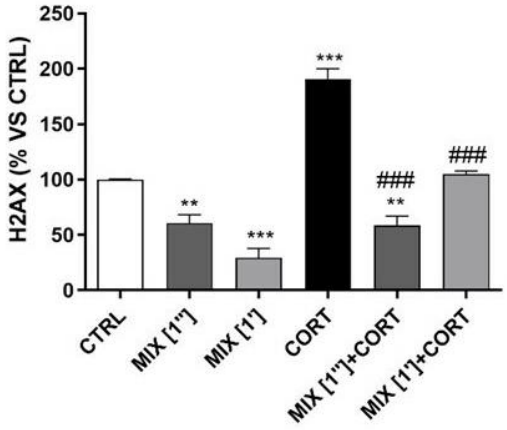

Figure 7. Evaluation of DNA damage. Following differentiation into cholinergic neurons, cells were treated with nutraceutical mix [1'’] and nutraceutical mix [1’] for 24 h and for an additional 24 h with cortisol at 250 µM. DNA damage was assessed by evaluation of histone H2AX phosphorylation levels. Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni’s post-test: ** $p<0.01$, *** $p<0.001$ vs control; ### $p<0.001$ vs cortisol.

3.9. Effect of the nutraceutical combination treatment on membrane fluidity

In the absence of antioxidant defenses, the predisposition in the brain to develop radical species [2] and oxidative stress, caused by non-physiological levels of cortisol [50], is responsible for the impairment of membrane fluidity. In this context, the effect of nutraceutical combination on membrane fluidity was analyzed. In the absence of cortisol, the mix had no influence on membrane fluidity; whereas, cortisol greatly reduced it, nutraceutical mix was able to prevent this reduction at [1'’] and [1’] concentrations (Figure 8), demonstrating its ability to protect the cell membrane from damage caused by cortisol-induced oxidative stress.

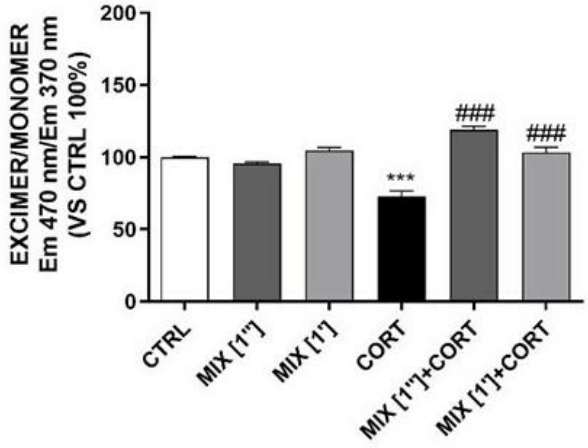

Figure 8. Evaluation of membrane fluidity. Following differentiation into cholinergic neurons, cells were treated with nutraceutical mix [1'’] and nutraceutical mix [1’] for 24 h and for an additional 24 h with cortisol at 250 µM. Membrane fluidity levels were assessed by fluorescence assay. Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni’s post-test: ** $p<0.01$ vs control; ### $p<0.001$ vs cortisol.

3.10. Effect of the nutraceutical combination treatment on aging-related genes

Alterations in the expression of genes such as brain-derived neurotrophic factor (BDNF) [51], cAMP response element-binding protein (CREB) [52], sirtuin (SIRT-1) [53] and plasticity-related gene 1 (PRG-1) have been related to different pathological mechanisms, including neuronal aging [54]. Challenging cholinergic neurons with the nutraceutical combination caused per se a significant enhancement in BDNF, SIRT1 and PRG-1 (Figure 9a,b,c,d). Cortisol decreased significantly the transcriptional levels of BDNF, SIRT1, CREB and PRG-1; the pre-treatment with the nutraceutical mix attenuated the cortisol-mediated decrease in the transcriptional levels of the examined genes, both at [1''] and [1'] concentrations (Figure 9a,b,c,d). These results may suggest that this nutraceutical combination effects on the induction of BDNF, CREB, SIRT-1 and PRG-1 gene expression may contribute to its cytoprotective effects in aged cholinergic neurons.

In order to study the effect of the mix on the induction of neurotrophic gene expression in a neural stem cell line, the H9-derived NSCs were used. Cortisol induced a significant reduction in the mRNA expressions of well-being-related factors (Supplementary Figure 6). Treatment with nutraceutical combination significantly prevented cortisol-induced effects, by reactivating the transcription of BDNF, CREB and SIRT-1 genes. The reduction in the expression of cortisol-induced genes, resulting in an increase in cellular well-being (Supplementary Figure 6).

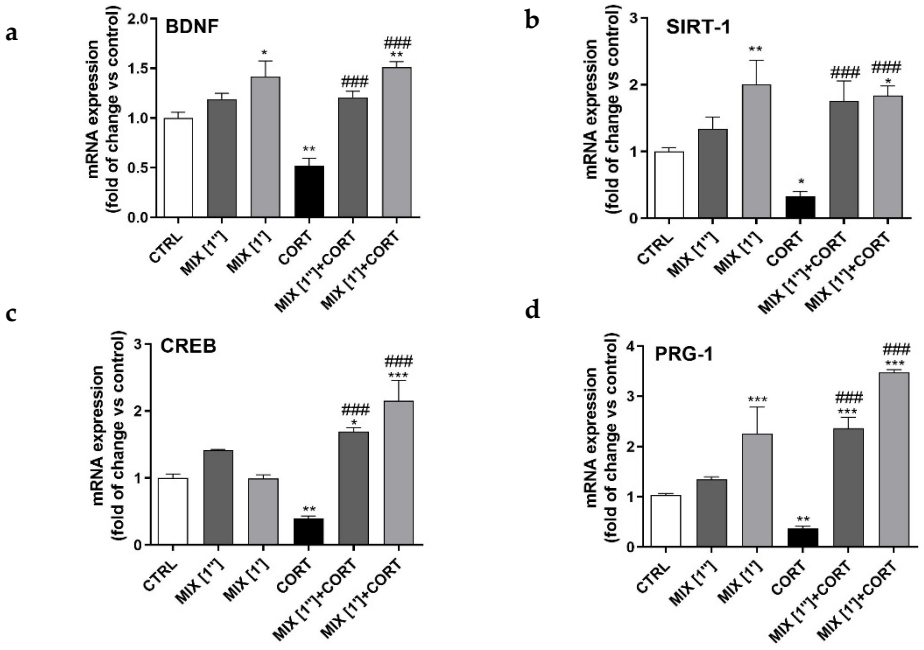

Figure 9. (a-d) Evaluation of genes expression related to well-being and neuronal plasticity. Following differentiation into cholinergic neurons, cells were treated with nutraceutical mix [1''] and nutraceutical mix [1'] for 24 h and for an additional 24 h with cortisol at 250 μM. At the end of the treatments, mRNA was extracted and the cDNA levels of BDNF (a), SIRT-1 (b), CREB (c) and PRG-1 (d) were quantified by real-time PCR analysis. Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentage of untreated cells set to 1 (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni's post-test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs control; ### $p<0.001$ vs cortisol.

3.11. Effect of the nutraceutical combination treatment on inflammatory molecules expression

During aging, the CNS inflammation process increases. In particular, COX-2 levels increase is generally closely related to ROS formation [55]. These observations lead to examining whether the nutraceutical combination reduces in turn COX-2 levels. In the absence of cortisol, [1'] concentrations significantly reduced COX-2 levels, proving to have an effect even on basal levels of the enzyme. As expected, cortisol induced an increase in COX-2, but the mix was able to restore COX-2 physiological levels (Figure 10a), demonstrating that it possesses a basic anti-inflammatory capacity and an ability to prevent an increase in inflammation induced by oxidative stress mechanism. To deepen nutraceutical combination anti-inflammatory function, IL-6 and IL-8 were also quantified. Both interleukins participate in neurogenesis processes and their expression is affected in several of the main brain diseases [56, 57]. As Figures 10b,c show, in the absence of cortisol, nutraceutical combination did not cause any change in IL-6 or IL-8 levels, with respect to control. In contrast, the increase of interleukin, induced by cortisol, was prevented by the mix at both concentrations, with a greater action at the highest concentration. These data demonstrate that the mix is able to reduce oxidative stress-induced inflammation.

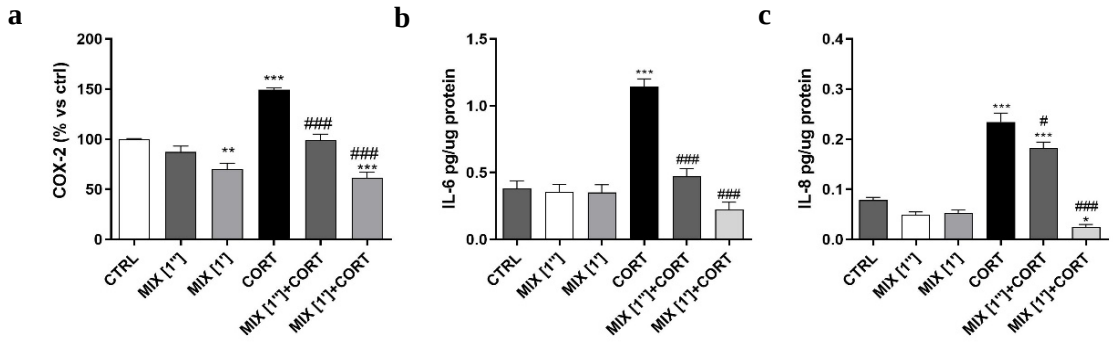

Figure 10. Evaluation of inflammatory signals. Following differentiation into cholinergic neurons, cells were treated with nutraceutical mix [1''] and nutraceutical mix [1'] for 24 h and for an additional 24 h with cortisol at 250 µM. COX-2 (a) and interleukins (b,c) levels were assessed by specific enzyme immunoassay. The data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni's post-test: ** $p<0.01$, *** $p<0.001$ vs control; $^{\#}$ $p<0.05$, $^{\#\#\#}$ $p<0.001$ vs cortisol.

4. Discussion

In the present study, neuroprotective properties of a nutraceutical combination were investigated in a model of cholinergic neurons. Specifically, the fix ratio of active compounds was demonstrated to have a protective role against mitochondrial dysfunction, inflammation and DNA damage. Moreover, challenging neuronal cells with this mix enhanced membrane fluidity and modulated overall gene transcription.

The effects of a specific ratio of omega-3 fatty acid, folic acid, vitamin E and magnesium on membrane and cellular well-being was verified human neuroblastoma cell line differentiated into cholinergic neurons. The cholinergic system is involved in learning, memory and age-related dementia [58] and represents a valuable cellular model for exploring the nootropic properties of compounds. For this purpose, cells were challenged with cortisol for 24 h in order to mimic physiological brain stress that can be related to aging [28]. In the present study, it was selected from a mix of several active nutraceuticals, known in literature to exert individually an activity in the physiology of the central nervous system, to test the possibility of a complementary and/or synergistic action. The fixed ratio of active ingredients present in the nutraceutical combination was initially chosen as they did not reduce cell viability by more than 20%.

First, to investigate the action of cortisol, an MTS and an apoptosis assay were performed. Consistent with our data, a cortisol treatment induced a decrease in HT22 cells viability and an increase in apoptosis [28], comparable to NaN_3 action in PC12 cells [39]. The mix significantly prevented cortisol- induced apoptosis. Consistently, EPA and DHA have been demonstrated to prevent cortisol-induced reduction in proliferation and increase in apoptosis in human hippocampal progenitor cell line [59]; at the same time, folic acid decreases astrocyte apoptosis by preventing oxidative stress-induced telomere attrition [60].

Assuming that high levels of cortisol regulate the expression of the ATP synthase genes at the mitochondrial level by reducing its expression [41], we challenged cholinergic neurons with cortisol and examined the ATP and ADP levels by calculating the ATP/ADP ratio. The results showed that nutraceutical combination significantly prevented the cortisol-mediated reduction of ATP and decrease in cell viability, at [1’] and [1’’] concentrations. Notably, these effects were mostly observed when the nutraceutical mix was administered following cortisol, thus evidencing the ability of the mix in restoring cortisol-induced energy imbalance and reverting its cytotoxic effects. Moreover, the effects displayed by the nutraceutical mix on ATP/ADP content were significantly higher with respect to those elicited by the single component, i.e., EPA/DHA, folic acid, vitamin E or magnesium, thus suggesting that additive/synergic effects may occur between the different nutraceutical components. Accordingly, literature reports similar synergistic cytoprotective or preventive effects of magnesium and Vitamin E [61], folic acid and vitamin B12 [62]. Among these papers, of particular relevance is the recent positive interaction of omega-3 fatty acid and vitamin E supplementation in improving working memory in older adults [63].

It is not clear why the mix [1 '] greatly increased the ATP/ADP ratio after the hydrocortisone application. These interesting results lead to hypothesize that, under physiological conditions, the supplement should not have particular effects on increasing ATP production, because the cellular energy request has been already satisfied. When the cell is in a state of prolonged stress, it is probable that the higher concentration of the nutraceutical combination has a greater capacity (compared to the concentration [1 "]) to restore the cellular basal conditions, due to an activation of several targets by the nutraceuticals. Consistent with our data, it has been reported that omega-3 supplementation delays age-related mitochondrial dysfunction in the brains of young and elderly mice [64]. In addition, by increasing the PGC1α and SIRT1 gene expressions, co-treatment with omega-3 and vitamin E has beneficial effects in patients with coronary artery disease, leading to an increase in ATP level [65]. On the other hand, magnesium stimulates the activity of several mitochondrial enzymes, and it is responsible for transporting ATP from mitochondria to the cytosol, which is mediated by an ATP-Mg / Pi carrier [11]; in particular, treatment of LmnaG609G/+ mice with dietary magnesium increased H+-coupled mitochondrial NADPH and ATP synthesis [66].

Following reduction of ATP levels, a serine/threonine kinase is activated [43], triggering autophagy processes [44]. In the absence of cortisol, nutraceutical combination treatment was able to reduce autophagy markers; similarly, following stimulus with cortisol, nutraceutical ingredients were able to significantly decrease the autophagy process at both concentrations. A considerable reduction was evident at [1’] concentration probably because higher mix concentrations favored the restoration of a cellular well-being environment, thus blocking autophagy. The physiological role and limits of autophagy are still debated in the current literature. This process is essential for maintaining proper cellular function and its dysfunction is associated with cancer, neurodegeneration, and aging [67]. Several works have demonstrated that mTOR inhibition by omega 3 induces autophagy processes, thus protecting cells from oxidative insults [68]. Conversely, other papers have demonstrated that antioxidant agents, including vitamin E, reduce autophagy markers in male Sprague-Dawley rats, relieving diabetic nephropathy [69], and that folic acid contrasts autophagosome formation in fatty liver disease rats [70]. In our hands, the presence of vitamin E and folic acid in the tested supplement, indeed composed by five-nutraceuticals, probably activates intracellular mechanisms that overcome omega 3-associated pathways, finally reducing the autophagic processes in our cellular model. In conclusion, we might speculate that the reduction of the autophagic process, in the absence of cortisol, could represent a beneficial mechanism. In conditions of oxidative stress, triggered by cortisol, nutraceutical combination exerted cytoprotective effects by inducing cellular autophagy.

During aging, prolonged exposure to high levels of glucocorticoids [5] in combination with low antioxidant defenses [6], can induce reactive oxygen species leading to oxidative damage in the CNS [38]. Challenging cholinergic neurons with nutraceutical combination significantly prevented the cortisol-induced ROS levels and DNA damage, both at [1’’] and [1’] concentrations. Notably, as evidenced for the ATP/ADP ratio, these effects were mostly maintained when the nutraceutical mix was administered following cortisol, thus evidencing the ability of the mix in restoring cortisol-induced imbalance in ROS production. Consistent with our data, omega-3 suppressed ROS formation in cultured neurons and neuronal progenitor cells of PPT1-free mice [71]; vitamin E protected cortical neurons against oxidative stress [72] and folic acid stabilized DNA, reducing damage [9]. Simultaneously, magnesium suppresses ROS production in various tissues, leading to the formation of magnesium–oxygen species [11]. Moreover, the effects displayed by the nutraceutical mix on ROS production were significantly higher with respect to those elicited by the single component, i.e., EPA/DHA, folic acid, vitamin E or magnesium. Similarly, Magnesium and Vitamin E co-supplementation have demonstrated a positive interaction on biomarkers of inflammation and oxidative stress in women with Polycystic Ovary Syndrome [61] and vitamin E has been proven to increase the biological effects of omega-3 fatty acids on redox balance in naturally aged rats [73].

In conditions of acute stress, the cell antioxidant mechanisms were responsible for restoring cellular well-being. One of the most important pathways is the Keap1/NRF2/ARE complex; under quiescent conditions, Nrf2 is anchored in the cytoplasm through binding to Keap1, which, in turn, facilitates the ubiquitination and subsequent proteolysis of Nrf2. Under stressful conditions, Nrf2 detaches from Keap1, translocates to the nucleus and activates a specific promoter sequence [74]. In contrast, in conditions of chronic stress, the Nrf2 pathway fails due to its degradation, depriving the cell of important antioxidant defenses [47]. Consistent with the latter evidence, challenging cholinergic neurons with cortisol for 24 h, induced a decrease in Nrf2 and Keap1 levels. Interestingly, the nutraceutical combination was able to prevent the cortisol-associated decrease in Nfr2 and Keap1 levels. These data are of particular relevance considering that Nfr2-Keap1 signaling has been linked to protective mechanisms against oxidative stress and proteotoxicity in cells, including neurons [75]. Similarly, omega-3 has demonstrated to improve the antioxidative defense in rat astrocytes and peritoneal macrophages via an Nrf2-dependent mechanism [12].

Brain aging is characterized by gene expression dysregulation of factors that influence neuronal plasticity and metabolism [1]. For this reason, we analyzed the expression of the CREB, BDNF, SIRT-1 and PRG1 genes [52, 51, 53, 54] in the SHSY-5Y cell line differentiated to cholinergic neurons and in the H9-derived NSC, in order to understand if the nutraceutical combination was able to regulate gene transcripts. These results demonstrated that the mix increases gene transcriptions of the mentioned transcripts, at [1’’] or [1’] concentrations and were equally able to prevent the reduction in cortisol-induced gene expression. Consistent with our data, omega-3 normalizes BDNF and CREB levels after traumatic brain injury in rats [76] and vitamin E enhances BDNF-TrkB-CREB signaling pathway in rats with cognitive impairment [77]. At the same time, omega-3 and vitamin E increase gene expression of SIRT1 and improve the response to oxidative stress in patients with coronary artery disease [65]. To date, no data in literature explored omega-3 or vitamin E effect on PRG1 expression. The lack of data on the action of folic acid on the expression of genes such as BDNF, CREB and SIRT1 could lie in the fact that its function is to prevent the accumulation of DNA damage in neurons caused by oxidative stress [78].

As a last evaluation step, the anti-inflammatory effects were tested in cholinergic neurons subjected to oxidative stress with cortisol. It is known that, during aging, CNS inflammation increases [5], therefore, COX-2 levels were evaluated as a value parameter linked to the presence of free radicals [79]. Challenging cells with the nutraceutical combination prevented cortisol-induced increase in inflammation. Consistent with our data, omega-3 is reported to show anti-inflammatory properties in rats with global ischemia [80], vitamin E inhibits COX-2 activity in human adenocarcinoma cells [81] and folic acid inhibits the proinflammatory action of COX-2 in rats given nicotine [82].

To deepen nutraceutical combination anti-inflammatory function, IL-6 and IL-8 were also quantified. Although neuronal cells are not largely deputed for the release of cytokines and pro-inflammatory molecules, literature reports that cytokines are proteins secreted in the central nervous system by microglia, astrocytes and

infiltrating peripheral immune cells and neurons too, under physiological and pathological conditions [83]. In the brain, cytokines are constitutively expressed in various brain regions by activated glial and neuronal cells [84, 34] and are involved in several normal and pathological processes including neuronal development, modulation of neurotransmitter metabolism and synaptic plasticity.

Challenging cells with the nutraceutical mix prevented cortisol-induced increase in IL-6 and IL-8 levels, with greater effectiveness at [1'] concentration. Consistent with our data, omega-3 were able to reduce inflammatory interleukins in a model of endothelial cells [85].

5. Conclusions

In human cholinergic neuronal-like cells, the nutraceutical combination was proven to: (i) prevent and revert the oxidative stress, energy imbalance and viability reduction induced by cortisol; (ii) prevent cortisol-induced DNA damage and disbalance in autophagy mechanism; (iii) increase membrane fluidity and transcription of genes related to cellular well-being; (iv) exert an anti-inflammatory effect by preventing COX2, IL-6 and IL-8 accumulation. Overall, these results confirm the complementary beneficial effects of simultaneous administration of omega-3, vitamin E, folic acid, and magnesium suggesting this nutraceutical combination as promising agent to preserve cholinergic neurons. In this sense, a recent report suggests the benefits of a combination of magnesium, folic acid, omega-3 fatty acids and vitamin E as a food supplement to complement brain functioning [86].

Author Contributions: E.Z. carried out biological experiments, analyzed the data and wrote the manuscript; S.D. was involved in scientific supervision and manuscript revision; L.C. and E.C. performed experiments and analyzed the data; C.M. was involved in funding acquisition and project supervision; M.V. was involved in scientific contribution and manuscript revision; G.M. was involved in project management, scientific contribution, and manuscript revision; L.D. was involved in scientific supervision, L.R. was involved in project supervision. All authors have read and agree to the published version of the manuscript.

Funding: This research was funded by Angelini Pharma S.P.A.

Declarations of interest: none.

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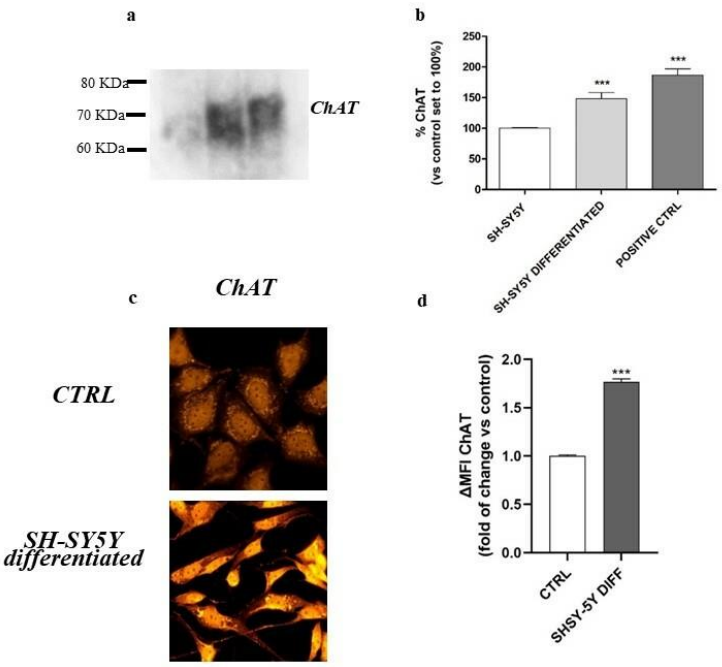
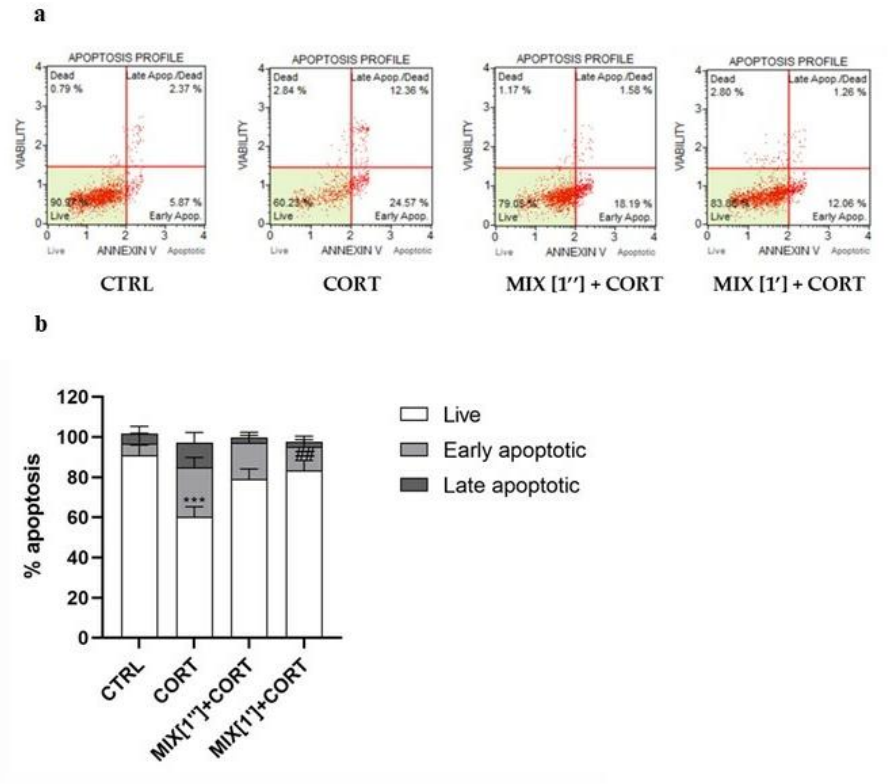
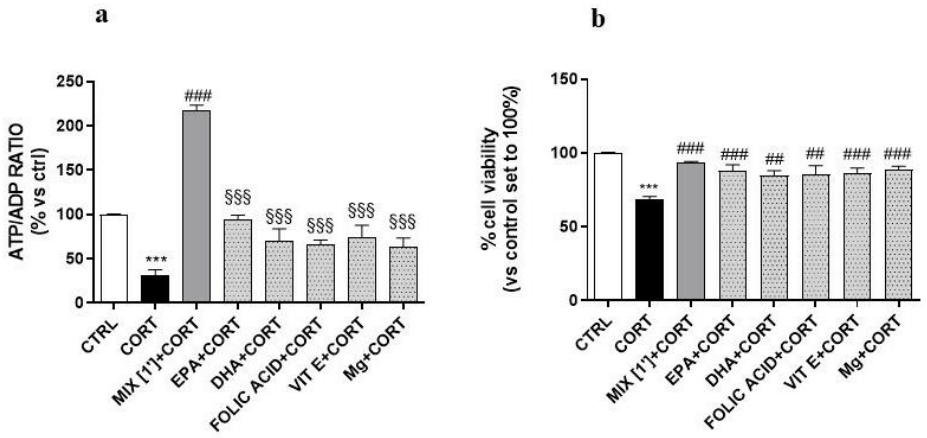
Supplementary material



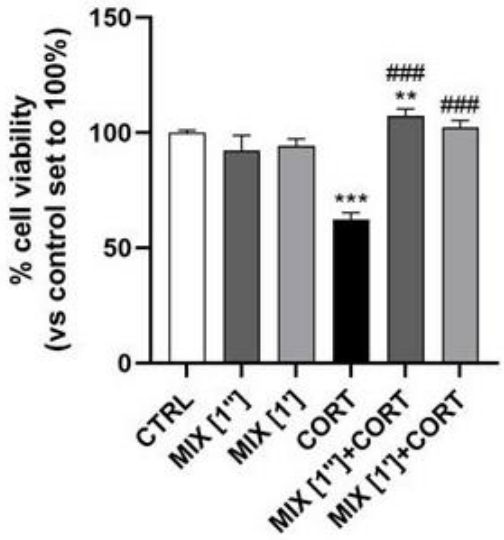
Supplementary Figure 1. (a–d) Differentiation of the human glioblastoma cell line SH-SY5Y into cholinergic neuronal cells. The SH-SY5Y cell line was differentiated into cholinergic neurons using 10 µM retinoic acid (RA) for 4 days and 50 ng/ml BDNF for the next 3 days. Following incubation, cells were collected and lysed. The expression of choline acetyltransferase (ChAT) enzyme was detected by Western blotting analysis (a,b). A representative Western blotting is shown (a). Irrelevant parts of the gel image are deleted. Optical density was measured by Image Lab software (b). Data are reported as percentage of control cells set to 100%. Immunofluorescence illuminates neuronal features of fully differentiated SH-SY5Y cells: anti-ChAT in red (c). Images were obtained at 60X magnification using an inverted epifluorescence microscope and they are expressed like mean fluorescence intensity (d). Data are the mean ± SEM of three different experiments, each performed in duplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni's post-test: *** $p<0.001$ versus control.



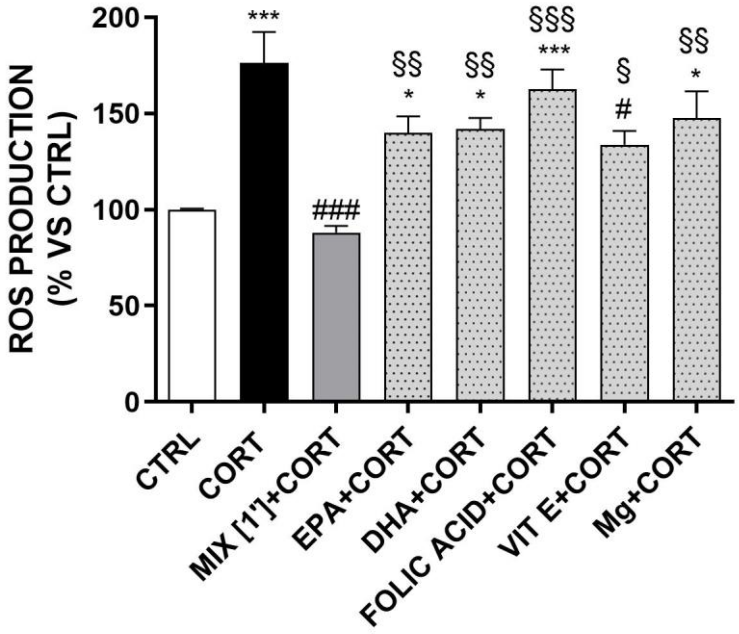
Supplementary figure 2. (a,b) Evaluation of apoptosis and nutraceutical combination effects. The SH-SY5Y cell line was differentiated into cholinergic neurons and after they were treated with the mix [1'']and mix [1'] for 24h and for an additional 24 h with cortisol at 250 µM. Following incubation, cellular apoptosis was measured by Muse Apo Assays (Merck-Millipore) as previously reported (22). Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni's post-test: *** $p<0.001$ vs control; $^{\#\#}$ $p<0.01$ vs cortisol.



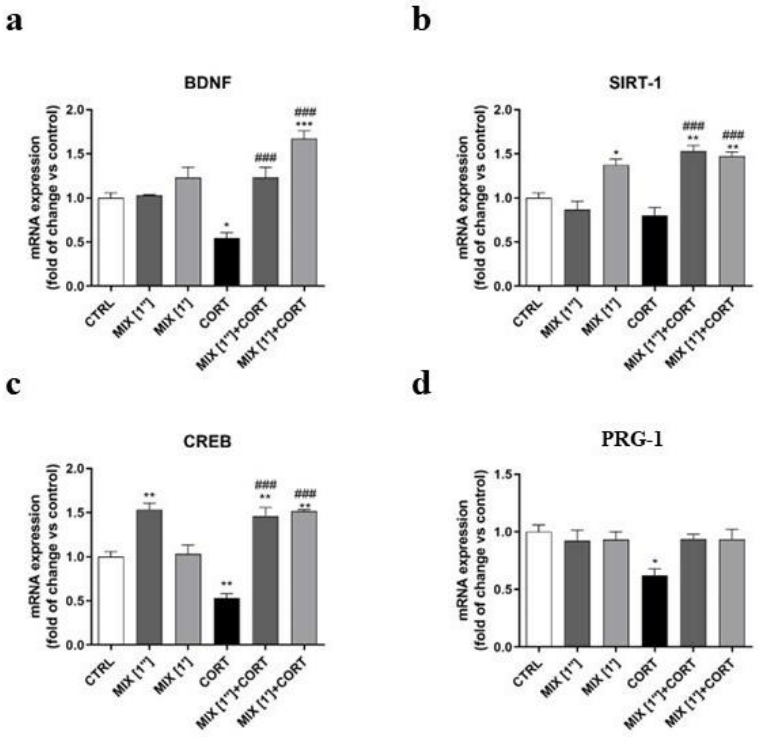
Supplementary figure 3: Evaluation of energy balance and cell viability for the single component of the nutraceutical mix. SHSY-5Y differentiated cells were treated with the nutraceutical mix, or with 8 μM EPA, 3.12 μM DHA, 4.5 nM folic acid, 52 nM vitamin E and 2.8 μM magnesium for 24 h and for an additional 24 h with cortisol at 250 μM; after treatments, the ATP/ADP ratio was evaluated by specific fluorometric assay (a), and cellular viability was measured by MTS assay (b). Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni's post-test: *** $p<0.001$ vs control; $^{\#\#}$ $p<0.01$, $^{\#\#\#}$ $p<0.001$ vs cortisol; §§§ $p<0.001$ vs nutraceutical mix.



Supplementary figure 4: Evaluation of H9-derived NSC viability. Cells were treated with the mix [1'’] and L/P mix [1’] for 24 h and for an additional 24 h with cortisol at 250 µM; after treatments cellular viability was measured by MTS assay. Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as percentage of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni’s post-test: ** $p<0.01$, *** $p<0.001$ vs control; $^{###}$ $p<0.001$ vs cortisol.



Supplementary figure 5: Evaluation of ROS production for the single component of the nutraceutical mix. SHSY-5Y differentiated cells were treated with the nutraceutical mix, or with EPA/DHA, folic acid, vitamin E or magnesium 8 μM EPA, 3.12 μM DHA, 4.5 nM folic acid, 52 nM vitamin E and 2.8 μM magnesium for 24 h and for an additional 24 h with cortisol at 250 μM; after treatments, ROS production was evaluated by by H2DCFDA fluorometric probe. Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as the percentages of untreated cells set to 100% (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni's post-test: * <0.05, *** p <0.001 vs control; # p <0.05, ### p <0.001 vs cortisol; §p<0.05, §§p<0.01, §§§p<0.001 vs nutraceutical mix.



Supplementary figure 6: Evaluation of gene expression related to well-being and neuronal plasticity in H9-derived NSCs. Cells were treated with the mix [1'’] and mix [1’] for 24 h and for an additional 24 h with cortisol at 250 µM; at the end of the treatments, mRNA was extracted and the cDNA levels of BDNF (a), SIRT-1 (b), CREB (c) and PRG-1 (d) were quantified by real time PCR analysis. Data are the mean ± SEM of three different experiments, each performed in duplicate, and reported as percentage of untreated cells set to 1 (control). Statistical analysis was performed by one-way analysis of variance (ANOVA) with Bonferroni’s post-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control; ### $p < 0.001$ vs cortisol.

FIGURE 1

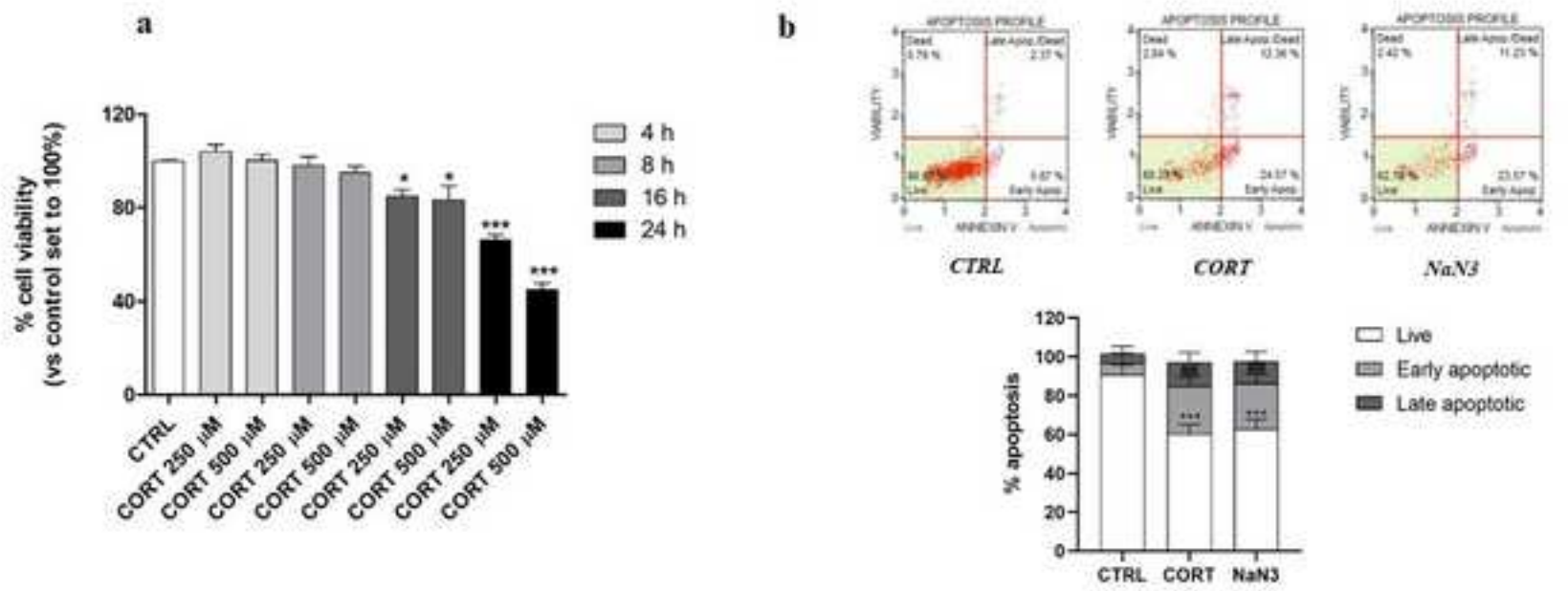


FIGURE 2

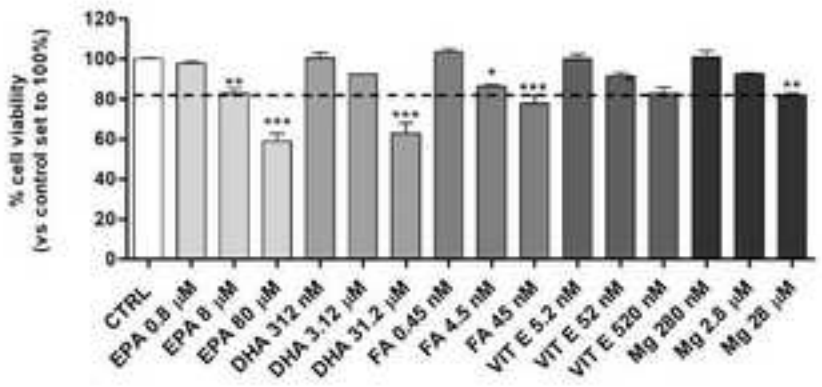
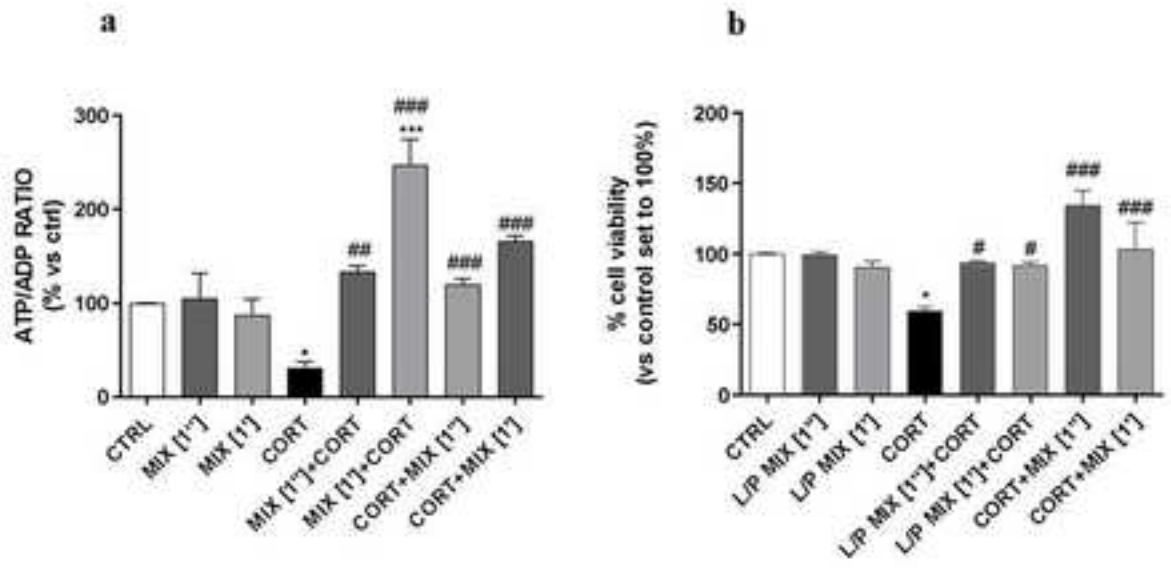


FIGURE 3



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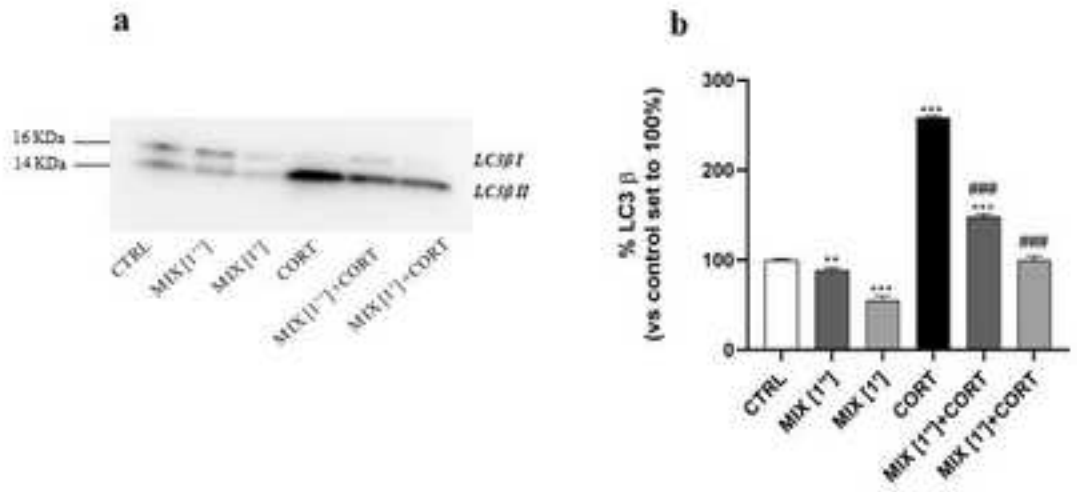

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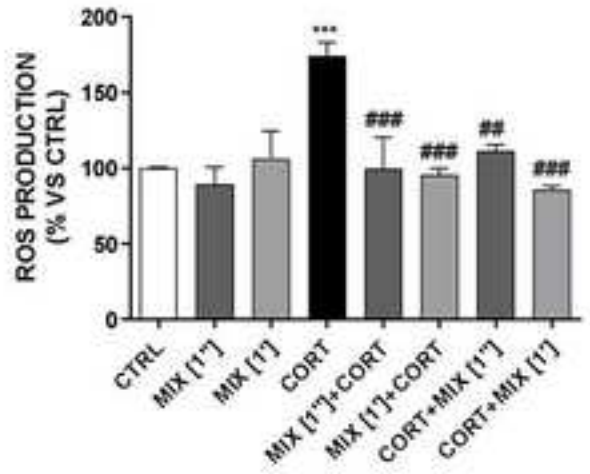

Figure 6

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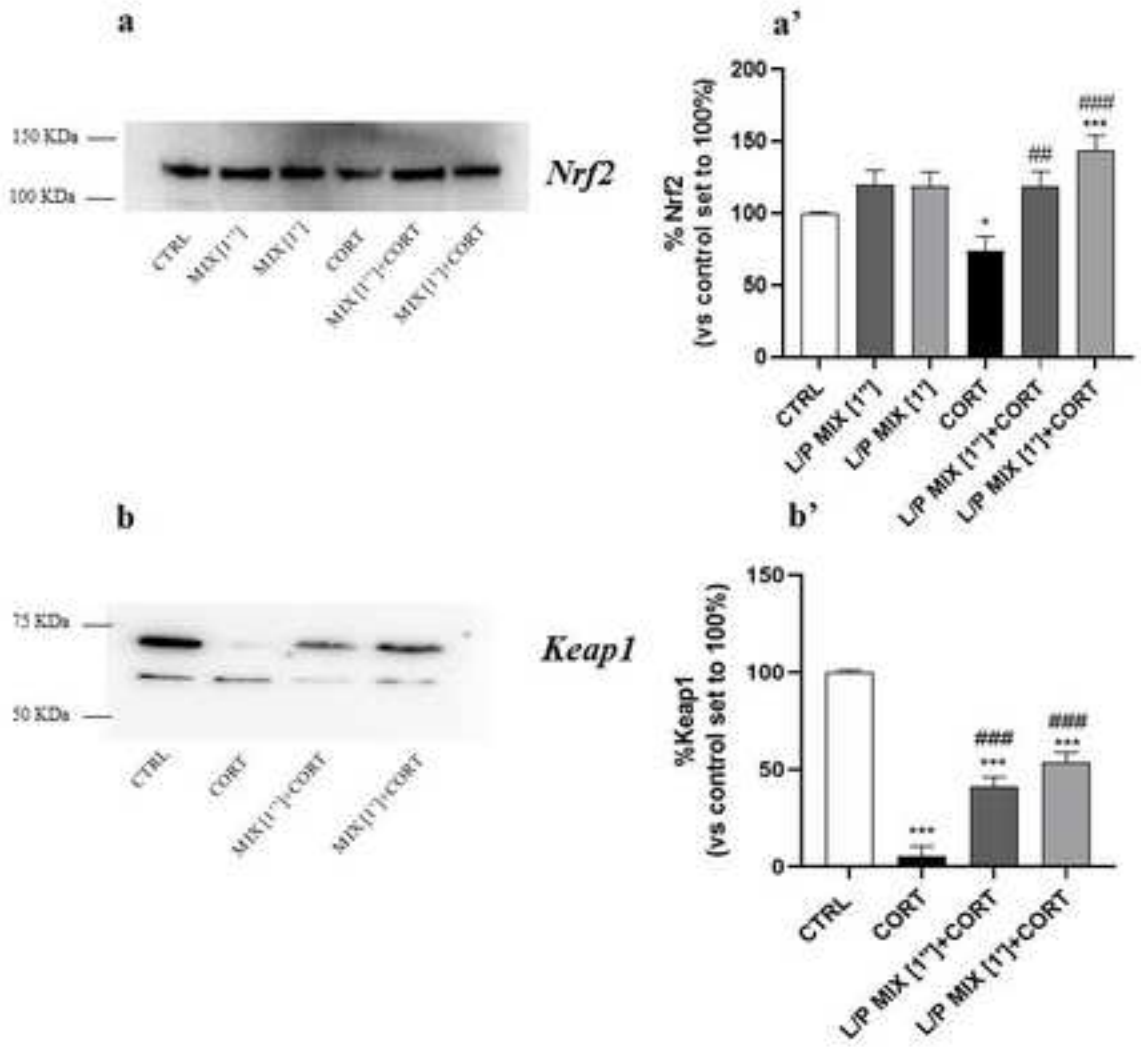


FIGURE 6

FIGURE 7

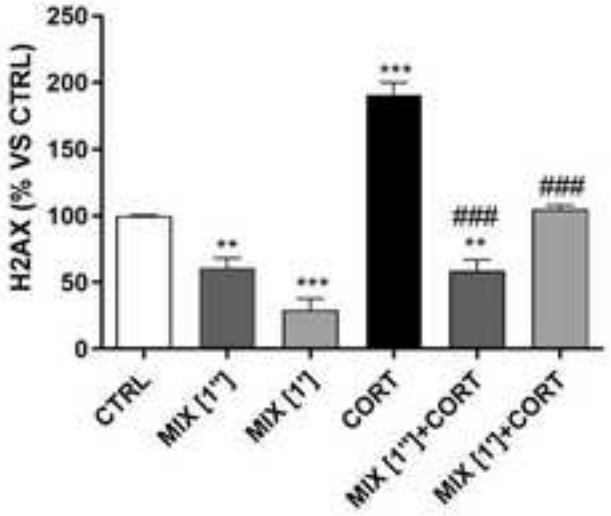
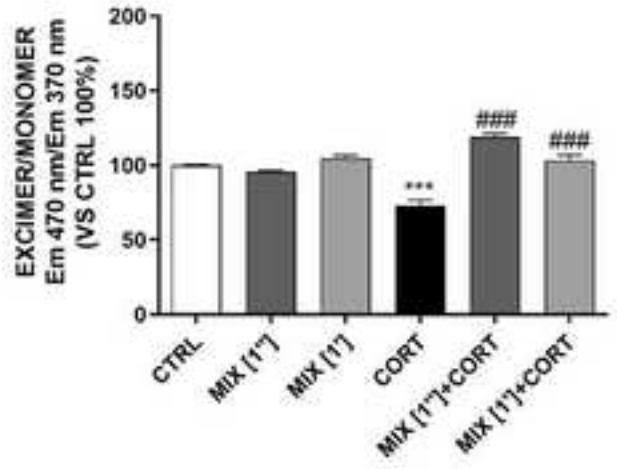


Figure 8

Click here to access/download;Figure;Diapositiva8.jpg

FIGURE 8



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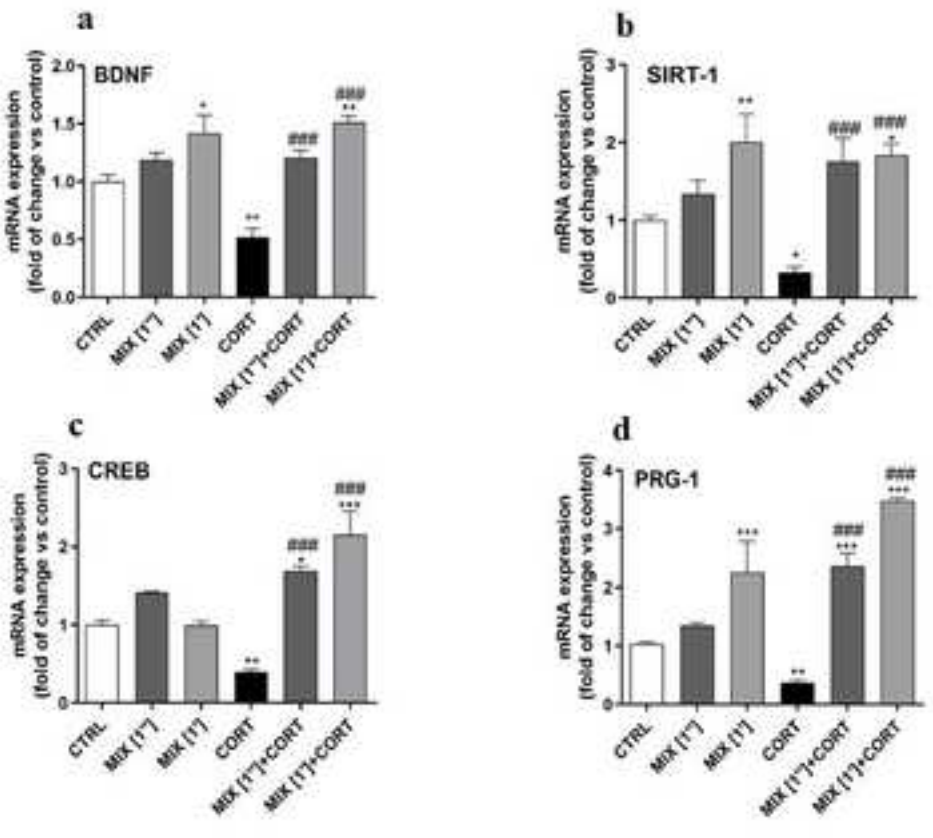
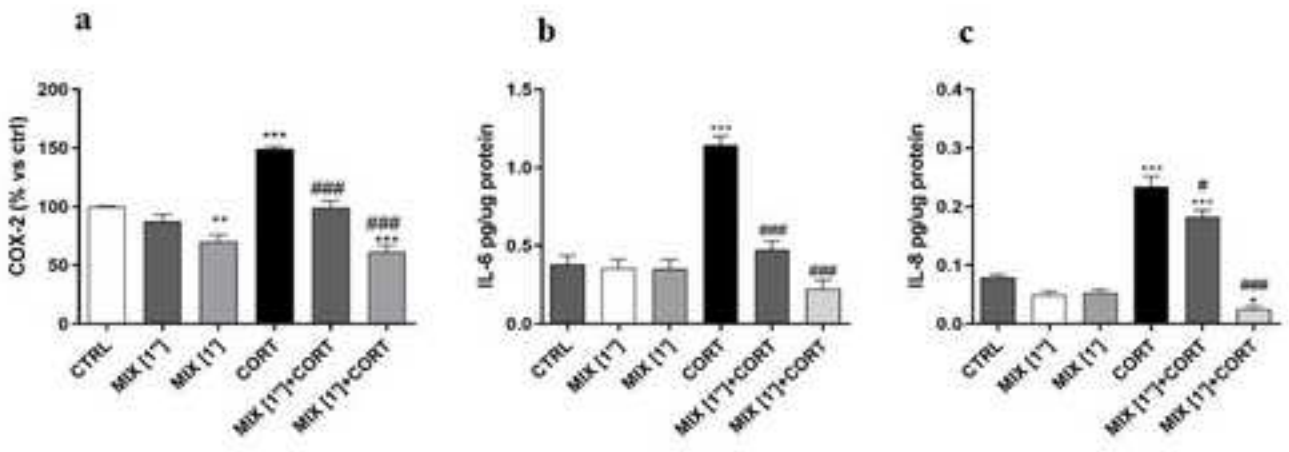


FIGURE 10



A Specific Combination of Nutraceutical Ingredients exerts cytoprotective effects in human cholinergic neurons.

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Declarations of interest: none