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Anti-neuroinflammatory effect of daidzein in human hypothalamic GnRH neurons in an in vitro membrane-based model

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Abstract

Phytoestrogens can control high-fat diet-induced hypothalamic inflammation that is associated with severe consequences, including obesity, type 2 diabetes, cardiovascular and neurodegenerative diseases. However, the phytoestrogen anti-neuroinflammatory action is poorly understood. In this study, we explored the neuroprotection mediated by daidzein in hypothalamic neurons by using a membrane-based model of obesity-related neuroinflammation. To test the daidzein therapeutic potential a biohybrid membrane system, consisting of hfHypo GnRH-neurons in culture on PLGA membranes, was set up. It served as reliable in vitro tool capable to recapitulate the in vivo structure and function of GnRH hypothalamic tissue. Our findings highlighted the neuroprotective role of daidzein, being able to counteract the palmitate induced neuroinflammation. Daidzein protected hfHypo GnRH cells by down-regulating cell death, proinflammatory processes, oxidative stress, and apoptosis. It also restored the proper cell morphology and functionality through a mechanism which probably involves the activation of ERβ and GPR30 receptors along with the expression of GnRH peptide and KISS1R.

KEYWORDS

daidzein, human GnRH neurons, hypothalamic neurons, neuoroinflammation, PLGA membrane system

1 | INTRODUCTION

Recent interdisciplinary studies in neuroscience have found a close relationship between overnutrition, especially high-fat diets, and inflammation in the brain, and particularly in the hypothalamus. The neuroinflammation causes a progressive damage of the neurons that control food intake and energy homeostasis, thus, leading to obesity, diabetes, and cardiovascular complications. Overnutrition-induced hypothalamic

Abbreviations: DCA, dynamic contact angle; DCF, dichlorofluorescein; DMSO, dimethyl sulfoxide; DZ, daidzein; ECM, extracellular matrix; ER, estrogen receptor; GnRH, gonadotropin releasing hormone; GPR-30, G-protein coupled receptor 30; hfHypo, human fetal hypothalamic cells; IL, Interleukin; KISS1R, kisspeptin receptor; LSCM, Laser Scanning Confocal Microscopy; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; NeuN, neuronal nuclei; PA, palmitate; PLGA, Poly(D,L-lactide-co-glycolide); PSCDs, Polystyrene culture dishes; ROS, reactive oxygen species; SEM, scanning electron microscope; SFAs, saturated fatty acids.

inflammation is also linked to neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and stroke.[1] Therefore, the investigation of potential anti-inflammatory agents that protect the hypothalamus from diet-induced inflammation represents an active area of research in the biomedical field.

Accumulating evidence suggests that intracellular disturbances and stresses including endoplasmic reticulum (ER) stress, oxidative stress, and autophagic defects are important mediators of hypothalamic inflammation. However, it is still needed to highlight the multiple mechanisms implicated in the induction of obesity-related neuroinflammation. Studies have demonstrated that elevated levels of saturated fatty acids (SFAs), such as the highly prevalent palmitate (PA), induce a state of neuroinflammation, characterized by several metabolic and molecular changes and severe damages in hypothalamic neurons. The accumulation of PA and its metabolites in the hypothalamus has been shown to increase the mRNA expression of pro-inflammatory cytokines, chemokines, and reactive oxygen species (ROS), and can result in apoptotic cell death.[2,3] Nevertheless, the effect of PA-associated hypothalamic inflammation on the different population of hypothalamic neurons is not fully understood.

The finding that estrogenic compounds provide neuroprotection against neuroinflammation although they have many adverse effects (e.g., increased risk of uterine, breast and endometrial cancer, heart disease and liver complications) raise the need to explore substitute compounds that have estrogenic properties without toxicities, as an alternative to estrogen replacement therapy.

Isoflavones are natural polyphenolic compounds, which act as phytoestrogens that have a diphenolic structure similar to estrogen with extensive pharmacological properties[4]; recently, their role as anti-obesity agents is emerging. Daidzein, 4′,7-dihydroxyisoflavone, a major isoflavone from soybean, is one of the most studied and potent phytoestrogens.[5,6] It has several activities such as antioxidant, anti-inflammatory, antitumor, and cardioprotective properties.[7–9] Previous studies have also reported the neuroprotective actions of daidzein against hypoxia in primary cortical neurons, excitotoxicity, β-amyloid, stroke-like injury, and ER stress-mediated neuronal degeneration,[10–12] besides to axonal outgrowth in neuronal cells. Daidzein regulates pro-inflammatory gene expression by activating PPAR-α and -γ and inhibiting the JNK pathway in adipocyte and macrophage co-cultures.[13] Recent evidences show the neuroprotective effect of daidzein in hypothalamic N42 cells against palmitic acid induced oxidative stress and apoptosis mediated by the transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator 1 alpha (PGC1-alpha), which plays a role in oxidative defense.[14] Despite these data, the exact mechanism of daidzein's neuroprotective effect has yet to be fully elucidated, specifically, the anti-neuroinflammatory action of daidzein in hypothalamic neurons is poorly understood and require further investigation. Indeed, considering the prominent role played by the fatty acids excess (palmitate administration) in the neuroinflammation induction[2,3] and the daidzein mediated wide protection spectrum,[7–14] it is reasonable to seek and highlight its capacity to stop and/or revers the inflammatory cascade.

To address this issue, the present work aims to elucidate the potential protective role of daidzein against palmitate-induced impairment in hypothalamic neurons that were cultured in a biohybrid membrane system in order to create an in vitro model of obesity-related neuroinflammation.

Previous works provided a proof of concept that semipermeable polymeric membranes are advanced biomaterials with a feature complexity, mimicking the native extracellular matrix (ECM), and topographical, chemical, and bio-functional cues that enable neuronal growth and differentiation.[15] Membranes, differently from other biomaterials that have been used to support neuronal cells cultures (e.g., electrospun, hydrogels, self-assembling peptides, nanoparticles, polystyrene dishes) display highly selective properties, which allow to create a fully controlled microenvironment at molecular level. These systems provide compartmentalization of cells governing the mass transfer of molecules to and from cells and offering appropriate structural and physico-chemical surface stimuli at nano and micro-scale, which are important for successfully supporting and guiding neuronal tissue repair and regeneration.[16–18] Membrane-based approaches have been used for the realization of valuable in vitro brain tissue models as investigational platforms for studying molecular mechanisms in physiological and/or pathological states including neurodegeneration,[19] drug testing,[20] and potential therapeutic strategy.[21,22]

However, there is still a gap regarding the development of an in vitro human neural tissue model. In the current study, we realized a novel highly selective membrane system for the culture of human hypothalamic neurons. We employed a primary culture of human fetal hypothalamic gonadotropin-releasing hormone (GnRH) neurons (hfHypo), previously isolated, and characterized, as cellular model whose functions have shown to be impaired by inflammation.[23] GnRH neurons are central regulators of the reproductive function and respond to inflammatory changes, thereby representing a crucial hypothalamic population implicated in the pathogenic mechanisms linking obesity to reproductive disorders.[24] As previously shown, hfHypo cell function is deeply affected by metabolic syndrome/obesity-related neuroinflammatory

cytokines, such as TNFα,[23] therefore, they represent a novel and valuable tool for investigating the direct effects of metabolic inflammatory factors in the human hypothalamus and to unravel the potential protective role of molecules, such as daidzein.

GnRH cell lines and other kinds of non-human immortalized cell lines have been used in some papers to study the effects of leptin, insulin and palmitate in the hypothalamus,[25,26] but the use of human primary GnRH neurons is limited. Moreover, there are no reports concerning the effects of daidzein on palmitate-induced neuroinflammation in hypothalamic neurons.

Hence, for the first time, we explored the anti-neuroinflammatory effects and mechanisms of action of daidzein in hfHypo neurons treated with palmitate. In this regard, we hypothesized that dadzein, thanks to its steroid structure, might activate, and exert neuroprotective effects in several settings that involve activation of estrogen receptors, as well as in regulating specific markers of neuronal differentiation and/or GnRH phenotype. We investigated its efficacy in promoting cell survival, in attenuating and/or suppressing palmitate-induced ROS production, mitochondrial dysfunction and apoptosis, and in retaining GnRH phenotype in vitro, in order to explore the possible underlying molecular mechanism.

The overall application represents also a promising resource to use membrane technology as preliminary drug screening in vitro tool for identifying new therapies to treat neurological disorders as well as for preclinical screening alternatively to animal experimentation.

2 | MATERIALS AND METHODS

2.1 | Membrane system: Preparation and characterization

Membranes of Poly(D,L-lactide-*co*-glycolide) (PLGA) were prepared in flat configuration by phase inversion technique. PLGA (7,5% wt/vol) (Resomer® RG 505, Mn 54.000–69.000, Sigma-Aldrich, Italy) was dissolved in dichloromethane. The polymeric solution was cast uniformly on the surface of a glass plate by using a handle-casting knife (Elcometer, gap set at 250 μm), dried at room temperature until complete solvent evaporation, and washed with distilled water. Then, the obtained PLGA membranes were characterized in order to evaluate their morphological, physico-chemical and mechanical properties that play an important role in guiding neuronal behavior.

Morphological properties of the PLGA membranes were characterized by scanning electron microscope (SEM). Samples of membrane surface and cross section were mounted with double-faced conductive adhesive tape, and, after gold coating, analyzed by SEM (ESEM FEG QUANTA 200, FEI Company) in order to evaluate surface and cross section structure, shape and sizes of the membrane pores.

The membrane thickness was measured with Carl Mahr 40E digital micrometer. Membrane mean pore diameter was determined using a Capillary Flow Porometer (CFP 1500 AEXL, Porous Materials Inc., PMI).

The physico-chemical properties of PLGA membrane ware characterized by water dynamic contact angle (DCA) measurements at room temperature by using a contact angle meter, CAM 200 (KSV Instruments, Ltd.).

Mechanical properties were evaluated by Zwick/Roell Z2.5 tensile testing machine. Membrane samples were cut into strips of 5×1 cm, mounted between two pneumatic grips, and subjected to uniaxial tension until failure. Ultimate tensile strength (UTS), Young modulus (Emod) and Elongation at break parameter (ε) were determined by analyzing five replicates of membrane sample.

2.2 | Cell culture and treatments

Hypothalamic GnRH neurons (hfHypo) were isolated from the brain of 12-week-old human fetuses as previously described,[23] and then cryopreserved. The use of human fetal biopsies for research purposes was approved by the National Ethics Committee and the local Ethics Committee for investigation in Humans of the University of Florence (Protocol Number: 678304) and was performed with the informed consent signed by the donors.

In the present work, hfHypo at passages P12–13 were seeded at a density of 16×10^3 cell/cm^2 on PLGA membranes in Coon's modified Ham's F12 medium (Euroclone, Milan, Italy) supplemented with 10% fetal bovine serum, and cultured under standard conditions (37°C, 5% CO_2). Polystyrene culture dishes (PSCD) were used as reference substrates. The previous work has shown the maintenance of the phenotype of this cell population until the passage P23.[23]

To realize an in vitro model of obesity-related neuroinflammation, neuronal cells after 48 h of culture in PLGA membrane system were exposed to sodium palmitate (PA, Sigma-Aldrich) at different concentrations (0–600 μM) for 24–48 h at 37°C. Palmitate was dissolved in water (50°C) to have a stock solution of 10 mM concentration and diluted in culture medium. The possible toxic effect of daidzein was evaluated by treating cells with different concentrations of molecule ranging from 0.1 to 10 μM.

To investigate the neuroprotective effects of daidzein (DZ, Santa Cruz Biotechnology) against palmitate-induced toxicity, cells were treated with a mixture of palmitate (300 μM) and daidzein (PA + DZ) at different concentrations (0.1, 1, and 10 μM) for 24 h at 37°C.

Daidzein was dissolved in DMSO to have a 10 mM stock solution and added to the culture medium at the indicated concentrations. We used the nomenclature "Control" to indicate untreated cells without palmitate and daidzein treatment. After 24 h under the different treatments, cells were sacrificed for morphological studies, and assessment of cell viability, neuronal behavior and functional responses, and the neuronal cell medium supernatant was collected and stored at −70°C for further analyses. All experiments were conducted in triplicate with the average of five samples for treatment (Control, Palmitate and PA + DZ).

2.3 | SEM analysis

The morphology of hfHypo-GnRH neurons was investigated by SEM analysis. All treated and untreated samples of neurons grown in PLGA membrane system were fixed with 2.5% glutaraldehyde for 30 min, followed by post-fixation in 1% osmium tetroxide and then dehydrated using a growing series of ethanol dilutions (10%–100%) and finally air-dried. Samples were examined at SEM and representative images displaying neuronal structural features were obtained.

2.4 | Immunostaining for laser scanning confocal microscopy

The behavior of hfHypo cells was analyzed by Laser Scanning Confocal Microscopy (LSCM, Fluoview FV300, Olympus) after the immunostaining of neuronal markers (β-Tub III and Neuronal nuclei, NeuN), GnRH phenotype markers (GnRH peptide and kisspeptin receptor, KISS1R) and estrogenic receptors (ER β and G protein-coupled receptor 30, GPR30). Neurons were fixed in 4% (wt/vol) paraformaldehyde for 30 min, followed by permeabilization and blocking with a solution containing 0.3% (vol/vol) Triton X-100 and 10% (vol/vol) FBS in PBS for 1 h at 37°C. Samples were then incubated overnight at 4°C with the following primary antibodies: anti-βTub III (1:500, Covance), anti-NeuN (1:200, Millipore), anti-GnRH (1:100, Abcam), anti-KISS1R (1:200, Santa Cruz Biotechnology), anti-ERβ (1:200, Santa Cruz Biotechnology) and anti-GPR30 (1:200, Abcam). Secondary antibodies, Cy2^{TM}-conjugated Affini Pure donkey anti-rabbit IgG, Cy3^{TM}-conjugated Affini Pure donkey anti-mouseIgG and a Cy5^{TM}-conjugated Affini Pure donkey anti-goat IgG (1:500, Jackson ImmunoResearch Europe Ltd.) were then added for 1 h at RT. Finally, cells were counterstained with 200 ng/ml DAPI (Molecular Probes), for nuclear localization.

2.5 | Measurement of cell viability

Cell viability was measured by 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay (Sigma-Aldrich). After 24 h of treatments, the culture medium was replaced with serum-free medium containing 5 mg/ml of MTT and incubated for 2 h at 37°C. Then, lysis buffer (10% SDS, 0.6% acetic acid in DMSO, pH 4.7) was added to dissolve formazan crystals, and the absorbance was measured with a spectrophotometer at a 570 nm wavelength. Cell viability is reported as percentage relative to control (100%).

2.6 | Cytokine determination

Cytokine production was assessed by analyzing the concentration of specific interleukins: Interleukin-6 (IL-6) and Interleukin-10 (IL-10), which were quantified by performing enzyme-linked immunosorbent assay (ELISA) in the supernatants of neuronal cultures.

Specific detection of IL-6 and IL-10 concentrations were obtained by a quantitative sandwich enzyme immunoassay technique.

A specific IL-6 ELISA kit (AbFrontiers) was employed for the quantitative detection of the IL-6 released by cells in the culture media, by following the manufacturer's protocol.

Monoclonal antibody specific to IL-10 (Thermo Scientific) was pre-coated onto a 96-well plate. Standards and cell culture supernatant were added to the wells with biotin-conjugated monoclonal antibody specific for IL-10. Therefore, all IL-10 was captured by the immobilized antibodies. Following a wash to remove any unbound substances, horseradish peroxidase-streptavidin conjugate was added to bind to the biotin on IL-10. Following another wash, a substrate buffer containing Tetramethylbenzidine (Thermo Scientific) was added to the wells. The reaction was stopped with 8 N H_2SO_4. Absorbance was measured at 450 nm using a Multiskan Ex (Thermo Lab Systems) and was proportional to the amount of IL-10.

2.7 | Cellular ROS detection assay

ROS generation in cells was measured using the probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Sigma-Aldrich), as previously described.[20] This molecule in presence of ROS is deacetylated and oxidized becoming the green fluorescent dichlorofluorescein (DCF). Then an increase in DCF fluorescence intensity is proportional to the ROS level. Neuronal cells (16×10^3 cell/cm^2) in the membrane system, after 24 h of treatment with

palmitate and the different concentration of daidzein, were loaded with 50 μM H_2DCF-DA at 37°C for 30 min. Then cells were washed twice with Hank's salt solution and the DCF fluorescence intensity was measured with LSCM, using an argon laser. Quantitative analysis was performed on confocal images of cells by using Fluoview 5.0 software (Olympus Corporation).

2.8 | Mitochondrial membrane potential measurement

The mitochondrial membrane potential of neurons was measured by using the fluorescent cyanine dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) (Sigma-Aldrich). JC-1 probe shows potential-dependent accumulation in mitochondria. An emitted red fluorescence (aggregates) indicates healthy mitochondria with normal potential, whereas the mitochondria with reduced potential emit green fluorescence (monomers). Therefore, the ratio of red to green fluorescence of JC-1 represents the condition of mitochondrial membrane potential. Cell cultures were incubated at 37°C with JC-1 (5 μg/ml) for 20 min. The fluorescence intensity of both monomers and J-aggregates were detected by LSCM. JC-1 green fluorescence was visualized with Ar laser and red fluorescence from JC-1 aggregates was detected using He/Ne green laser. Then, red/green fluorescence intensity was measured, and a quantitative analysis was performed on confocal images of cells by using Fluoview 5.0 software.

2.9 | Apoptosis assay

The apoptotic degree was evaluated by investigating the expression of two apoptotic markers, caspase-3 and phospho-JNK (p-JKN), which were detected by immunostaining with rabbit anti-caspase-3 antibody (1:250, BD) and mouse anti-p-JNK (Thr183/Tyr 185) antibody (1:250, Santa Cruz Biotechnology), respectively. Quantitative analysis of apoptotic cells was performed by calculating the percentage of positive cells for each marker (caspase-3 positive nuclei and p-JNK positive nuclei) over the total (DAPI-stained nuclei).

2.10 | Statistical analysis

Statistical significance of the experimental results was assessed using ANOVA test followed by Bonferroni *t*-test. The results were expressed as mean ± SEM from at least three independent experiments in which five samples for each treatment were assessed. A p value <0.05 was considered to be statistically significant.

3 | RESULTS

3.1 | PLGA membrane system sustains the growth of hfHypo neurons

PLGA membrane underwent to a full characterization in order to disclose their intrinsic properties that strongly impact on hypothalamic cell behavior in reorganizing themselves according to a proper structure. For a faithful tissue-like assembly, the appropriate cell displacement must be reproduced as a consequence of the correct interactions between cells and with the surrounding environment.

Flat PLGA membranes were morphologically characterized through SEM analysis revealing a homogenous and smooth surface, with nanopores regularly distributed over it (Figure S1).

Porometer measurements pointed out a mean pore size distribution of 20 ± 2 nm (Table 1). Membrane physical chemical properties directly influence cell-material interaction and therefore the overall tissue formation process. To better predict and evaluate membrane performance in terms of cell growth supporting ability, dynamic contact angle measurements were carried out. Surface characterization pointed out an advancing contact angle of 81° ± 3° and a receding one with a value of 44° ± 4°, evidencing a moderate wettability which is a desirable condition for promoting an optimal interaction at the cellular-material interface. When cells are cultured in vitro they can sense material rigidity, exhibiting a stiffness-dependent behavior on the basis of the mechanical features that characterize their natural niche. Biomechanical stimuli arising by the substrate are responsible of cell migration, polarization, and differentiation, representing therefore an important membrane characteristic

TABLE 1 Poly(D,L-lactide-*co*-glycolide) (PLGA) membrane properties

PLGA membrane	
Thickness [μm]	9.3 ± 1
Mean pore diameter [nm]	20 ± 2
WCA [°]	θ_{adv} 81 ± 3
	θ_{rec} 44 ± 4
E [N/mm^2]	315 ± 17
ε [%]	11 ± 0.8
UTS [N/mm^2]	7 ± 0.6

Note: Advancing $\boldsymbol{\theta_{adv}}$ and receding $\boldsymbol{\theta_{rec}}$ contact angle; Young's modulus **E**; elongation at break **ε**; ultimate tensile strength **UTS.**

that needs to be investigated. PLGA membrane exhibits a moderate stiffness as demonstrated by the Young's modulus value of 315 ± 17 N/mm^2 (see Table 1) in comparison with other materials utilized for neuronal cells that range from few hundred Pa (e.g., hydrogels) to 1–2 GPa (e.g., tissue culture polystyrene).

The PLGA membrane properties characterization was followed by its assessment in sustaining neuronal growth and differentiation. PSCD were used as reference substrate. This preliminary biological investigation is fundamental to validate our membrane system as reliable hypothalamic tissue model for daidzein neuroprotective screening. To prove this, hfHypo-GnRH neurons were cultured on PLGA membranes and on PSCD and underwent to morphological observation and evaluation. SEM analysis after 3 days of culture evidenced that cells established a proper interaction at the membrane interface, clearly visible by the high degree of adhesion and considerable portion of membrane surface covered by the cells (Figure 1A). On both kind of substrate, PLGA membranes (Figures 1A–C) and PSCD (Figures 1D–F), cells reached a structural maturation, acquiring a typical bipolar shape, where the two deriving unbranched processes are the connection to the other cells. At first glance, the cellular reorganization over the two substrates is quite similar, thus evidencing that the PLGA membrane system is comparable to the reference one.

GnRH–secreting neurons were stained for typical neuronal lineage markers, to further assess also the ability of the membrane system in addressing and boosting a full cellular differentiation. Images reconstruction was performed through a confocal microscope analysis (Figure 1B,C,E,F), which allowed to visualize the co-localization of distinctive neuronal markers, such as β-TubIII and NeuN. Representing one of the main neuronal cytoskeletal structural protein, β-TubIII is properly visible within the soma and along neuronal processes as indicator of an appropriate cell arrangement within the new microenvironment. NeuN was also present in the nuclei and perinuclear cytoplasm. The contemporary presence of these distinctive markers confirmed that neuronal cells went through the typical morphological changes of differentiation including axon sprouting and elongation, demonstrating also the good performance of the membrane system (Figure 1B). For a complete cellular analysis overview, a further immunocytochemistry investigation was carried out to characterize the neuronal hypothalamic phenotype, through the identification of the positive expression of specific indicators. To assess their nature and to confirm that they are GnRH neurons, hfHypo cells were simultaneously stained for KISS1R, the receptor for kisspeptin to which hypothalamic cell source are able to respond, and for the hormone GnRH. As reported in Figure 1C, confocal images showed the coexpression of these two markers corroborating that cultured cells have a GnRH-neuron phenotype. This evidence is also sustained by the positive immunolocalization for the sex steroids receptors ER-β

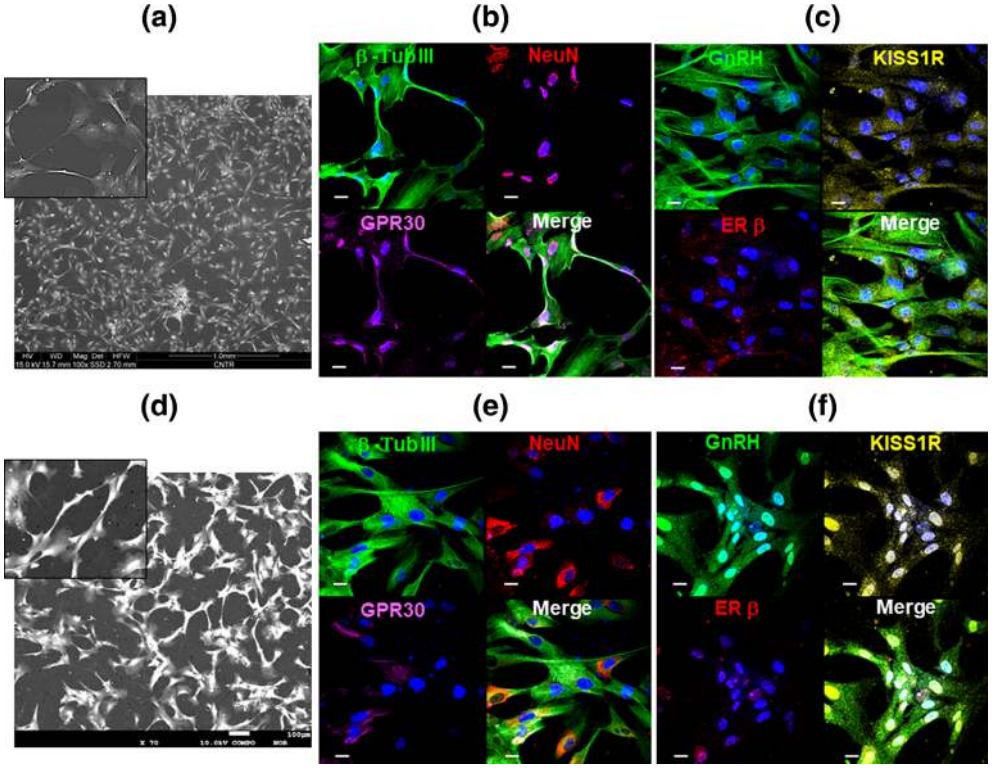


FIGURE 1 PLGA membrane system boosts hypothalamic GnRH neuronal growth. (A,D) SEM images at different magnification and (B–C,E–F**)** confocal laser micrographs of neuronal cells at DIV3 in PLGA membrane system (A–C) and in PSCD control system (D–F). Cells were stained for β-Tub III (green), NeuN (red), GPR30 (magenta), KISS1R (yellow), GnRH (green), ER β (red), and nuclei (blue). Scale bar in Confocal images is 20 μm

(Figure 1B) and GPR30 (Figure 1C). Both receptors are extensively visible being typically expressed by this peculiar family of neurons.

As expected the PSCD, being the extensively used reference system in cell culture studies, allowed the acquisition of a neuronal hypothalamic phenotype thus hfHypo cells expressed all investigated neuronal markers. In contrast, cells on PSCD were not able to express the estrogenic receptors, especially GPR30, at the same extent of those displayed on PLGA membrane system, as shown in Figure 1E, and the expression and distribution of GnRH (Figure 1F) was not wide as the one observed on PLGA membranes (Figure 1C). These evidences validated PLGA membrane system as reliable hypothalamic tissue model able also to boost the GnRH phenotypic peculiarity.

3.2 | Modeling neuroinflammation in the PLGA membrane system

Before assessing the neuroprotective effect of daidzein, it was necessary to set up an efficient disease model, where a realistic inflammatory neurological response evoked by a high-fat dietary intake was reproduced.

SFA palmitate was identified to play an important role as a mediator involved in the inflammation events that occur in obesity. Therefore, hypothalamic cells were exposed to different concentrations of palmitate. To identify the amount of palmitate that produced a 50% reduction in cell viability, cells were exposed to a concentration range of the fatty acid comprised between 0 and 600 μM and the effects evaluated at the time point of 24 and 48 h (Figure 2). After 24 h, cells exposed to the concentration of 100 μM of palmitate were still viable (98.6% ± 5%) with a profile quite similar to the control (100% ± 3.2%). Incubation with higher concentration of palmitate affected cell viability in a dose-dependent manner. A viability reduction of 52.9% ± 3.3% was observed for the concentration of 300 μM with further decline when cells were treated with higher concentration (400–600 μM). The prolonged exposure for 48 h reveals palmitate toxic effect already at the lowest concentration of 100 μM and a viability decrease to 55.2% ± 12%. A drastic reduction of cell viability to 6.5% ± 4%, at this time frame, is observed at the concentration of 300 μM, and this trend is kept constant with the higher concentration. These results highlighted cell response to palmitate time exposure, which significantly affected cell survival. The concentration of 300 μM for 24 h was identified as the reference one, and used to model in vitro neuroinflammation and to assess the neuroprotection mediated by daidzein co-exposure.

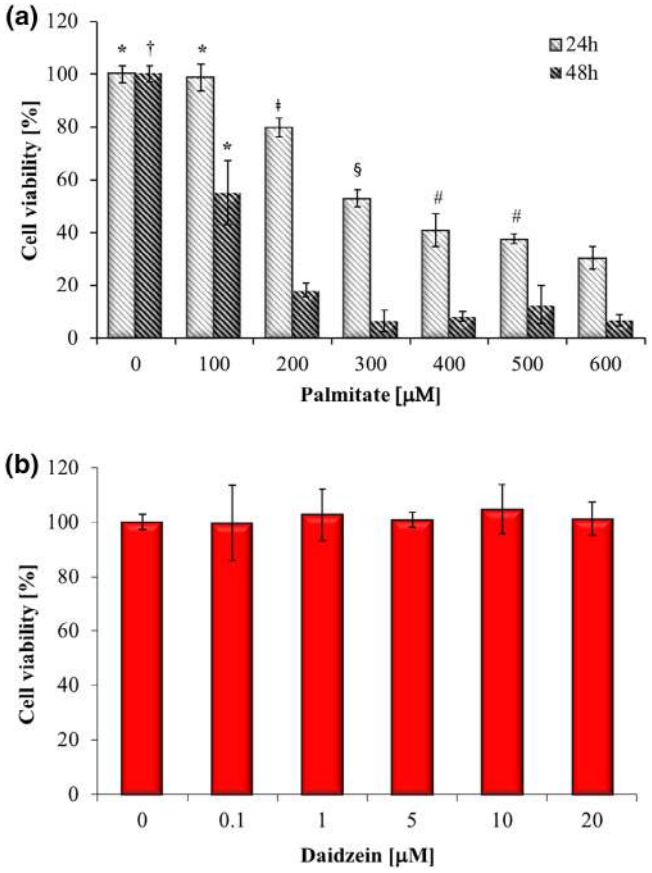


FIGURE 2 Viability of hfHypo GnRH neurons in the PLGA membrane system. (A) Dose-dependent toxic effects of palmitate on GnRH neuronal cells. Cell viability after 24 and 48 h of treatment with different concentrations of palmitate. The values expressed as average ± SD are the means of five experiments and data statistically significant were evaluated according to ANOVA followed Bonferroni *t*-test ($p < 0.05$). * versus palmitate 200–600 μM; ‡ versus palmitate 300–600 μM; § versus palmitate 400–600 μM; # versus palmitate 600 μM; † versus palmitate 100–600 μM. (B) Cell viability after 24 h of treatment with various concentrations of daidzein

3.3 | Daidzein protects GnRH cells against palmitate-induced cell death and reverses its inflammatory induced damage

To evaluate whether daidzein alone is toxic and influences neuronal cell viability, hfHypo cells were treated with various concentrations of daidzein (0–20 μM) for 24 h. As shown in Figure 2B, cells maintained high viability levels demonstrating that daidzein is not toxic for neuronal cells.

The first screening of daidzein neuroprotective effects included GnRH neurons morphological observation and their viability assessments, comparing cell response to different treatments: basal condition with culture media,

exposure to 300 μM palmitate, cells co-treatment of palmitate (300 μM) and daidzein at different concentration (0.1–1–10 μM).

SEM images reported in Figure 3A display that hfHypo cells kept in basal condition (I) adhered to the membrane and maintained their typical morphology; the induced damage, by exposing cells to 300 μM palmitate (II), caused cell loss and the few cells still adhered over the membrane surface exhibited a shrunken morphology with no projection and therefore a compromised neuronal cells communication. A reversed situation was visible when cells underwent to a co-treatment with the fatty acid and the daidzein (III-IV). Indeed, the acute palmitate treatment appeared totally neutralized by the simultaneous exposure to daidzein as demonstrated by the wide membrane surface area covered by the cells while keeping a proper morphology of healthy cells. The same trend was observed in terms of cell viability (Figure 3B) with the graph that clearly shows an increasing cell survival as increases the concentration of daidzein. When the phytoestrogen is used at the concentration of 1 and 10 μM the percentage of viable cells was 89% ± 7% and 101% ±6%, respectively, which were significantly higher than that obtained in cells insulted with palmitate (58% ± 5%). At the concentration of 10 μM, the viability percentage was totally restored with value equal to the control (100% ± 1%), indicating a full protection offered by the daidzein.

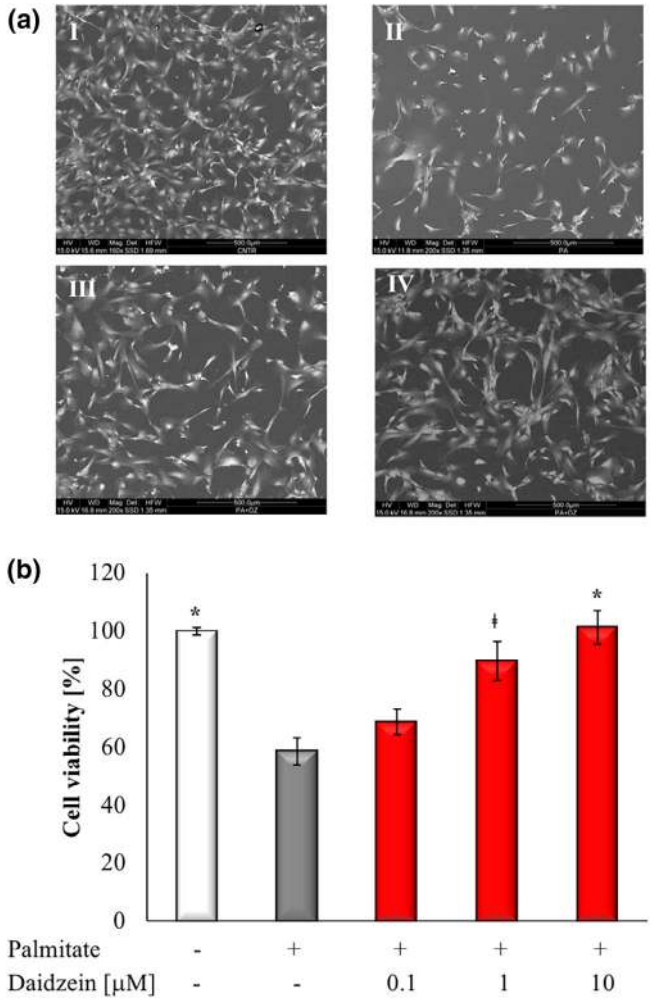


FIGURE 3 Daidzein protected GnRH cells against palmitate-induced cell death. (A) Representative images depicting the morphology of neuronal cells after 24 h of treatment with Palmitate (300 μM) and Daidzein at different concentrations; Control cells without any treatment (I); Palmitate (300 μM) treatment (II); co-treatment with: palmitate and daidzein 1 μM (III), palmitate and daidzein 10 μM (IV). (B) Viability of GnRH cells after treatment with of Palmitate (300 μM) and Daidzein at different concentrations (0.1–1–10 μM) for 24 h. The values are presented as percentages of control (no palmitate treatment). Data are mean ± SEM from three independent experiments and data statistically significant were evaluated according to ANOVA followed Bonferroni *t*-test ($p < 0.05$): * versus other treatments; ǂ versus palmitate 300 μM and Palmitate+ daidzein 0.1 μM

The obesity associated inflammation process, evoked in the GnRH cells through palmitate exposition and the cellular counteracting response, activated by the daidzein treatment, was investigated by quantifying the release of IL-6 and IL-10, reported in the graph in Figure 4A,B, respectively. Neuropathological changes are often associated with the increased IL-6 expression in brain, since this cytokine plays a pivotal role in the inflammatory response. Figure 4A depicts IL-6 release and as expected the palmitate treated cells delivered the highest amount of the pro-inflammatory cytokine (274 ± 26 pg/10^6cell) as a sign of the ongoing neuroinflammation process. The IL-6 production is gradually reduced when the palmitate inflammatory stimulus is accompanied by the co-treatment with daidzein. The graph highlights a progressive IL-6 reduction as the concentration of daidzein increases; the inhibition of IL-6 production is quite significant ($p < 0.05$) at the daidzein treatment of 10 μM as demonstrated by the reduction of IL-6 secretion at a value of 51.9 ± 11.3 pg/10^6cell, which is similar to that of the untreated cells (22.2 ± 4.3 pg/10^6cell). To further asses the daidzein anti-inflammatory properties, the IL-10 secretion was also analyzed. The anti-inflammatory cytokine IL-10 is responsible for balancing the immune response in the brain and therefore produced as a defense reaction to an inflammatory mediator. The ability of daidzein to reverse neuroinflammation was supported by the evidence in graph 4B in which the only palmitate treatment induces a peak of IL-10 (148.5 ± 23 pg/10^6cell), but its production is consistently attenuated by daidzein co-treatment in a concentration dependent manner.

The high IL-6/IL-10 ratio measured in the palmitate-treated cells (1.87 ± 0.34) decreased as the concentration

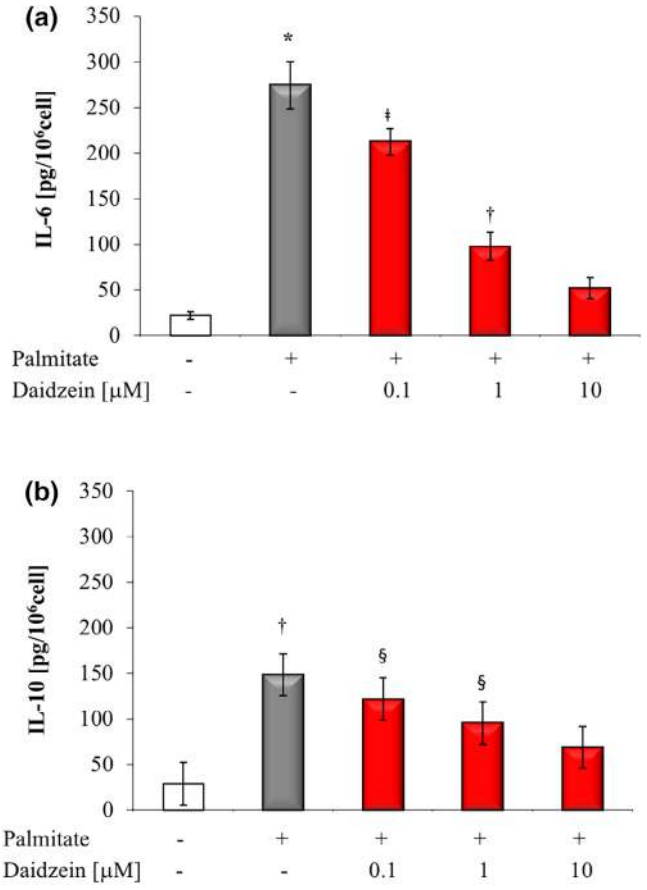


FIGURE 4 Anti-inflammatory role of Daidzein. Daidzein suppressed palmitate-induced cytokine secretion from GnRH neurons: IL-6 (A) and IL-10 (B) production. The values expressed as average ± SD are the means of three experiments and data statistically significant were evaluated according to ANOVA followed Bonferroni *t*-test ($p < 0.05$). * versus other treatments; ǂ versus control, palmitate + daidzein 1 μM, palmitate + daidzein 10 μM; † versus control and palmitate + daidzein 10 μM; § versus control

of daidzein increases, reaching value equal to the control (0.69 ± 0.47) when cells were treated with daidzein 10 μM (0.63 ± 0.17) (Table 2). These findings indicate that the overall inflammation process was silenced in response to the daidzein treatment.

3.4 | Daidzein neuroprotection is mediated by its antioxidant activity and anti-apoptotic effects

We explored if the neuroprotection, offered by daidzein to palmitate insulted cells, is evoked by its antioxidant effect. Therefore, to disclose this mechanism we proceeded to seek the ability of daidzein in preventing intracellular ROS accumulation. To this purpose, the H_2DCFDA probe was used to highlight the redox balance maintenance through daidzein activity. As shown in Figure 5A palmitate treatment led to a massive production of ROS as qualitatively evidenced by the bright green fluorescence compared with the control. Quantitative analysis of the green fluorescence emission, whose intensity is directly linked to the ROS production and accumulation, showed that daidzein co-treatment significantly reduced ROS production in a dose-dependent manner (Figure 5B). The stronger intracellular antioxidant activity was obtained at the daidzein concentration of 1 and 10 μM, quantifying a ROS intracellular in the same physiological range of normal cell metabolism.

To further characterize daidzein neuroprotective properties, its action in preventing the apoptotic cascade was also analyzed. To fulfill this task as first step was investigated the phytoestrogen potential to avoid mitochondrial dysfunction. Since mitochondria are inherently involved in the apoptotic process of cells, the JC-1 dye was used to monitor mitochondrial permeability; indeed, damaged cells undergo to a progressive pore opening with the consequent loss of the mitochondrial permeability potential (MMP), which is an intracellular event that occurs during apoptosis. The JC-1 staining was evaluated in GnRH neuronal cells kept in different culture conditions in order to assess if daidzein could protect cells from the palmitate-induced mitochondrial damage. As shown in Figure 6A when cells are treated only with palmitate (II) and co-treated with daidzein 0.1 μM (III) the green fluorescence signal increased, compared with the control and to the other samples co-treated with other concentrations of daidzein. The increase of the green fluorescence emission

TABLE 2 IL-6, IL-10, and IL-6/IL-10 ratio

Treatment	IL-6 [pg/10^{6}cell]	IL-10 [pg/10^{6}cell]	IL-6/IL-10 ratio
Control	22.2 ± 4.3	29.2 ± 18	0.69 ± 0.47
PA	274 ± 26	148.5 ± 23	1.87 ± 0.34
PA+ DZ 0.1 μM	212.6 ± 14.8	121.9 ± 22	1.79 ± 0.39
PA+ DZ 1 μM	97.6 ± 15.4	95.4 ± 21	1.02 ± 0.28
PA+ DZ 10 μM	51.9 ± 11.3	68.9 ± 23	0.63 ± 0.17

Note: Data are presented as mean ± SD.
Abbreviations: DZ daidzein; PA, palmitate.

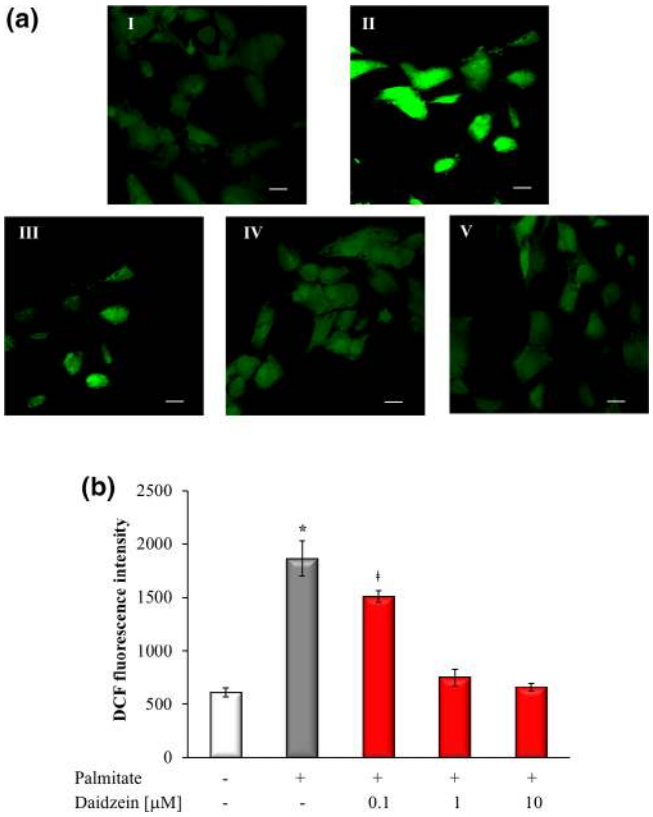
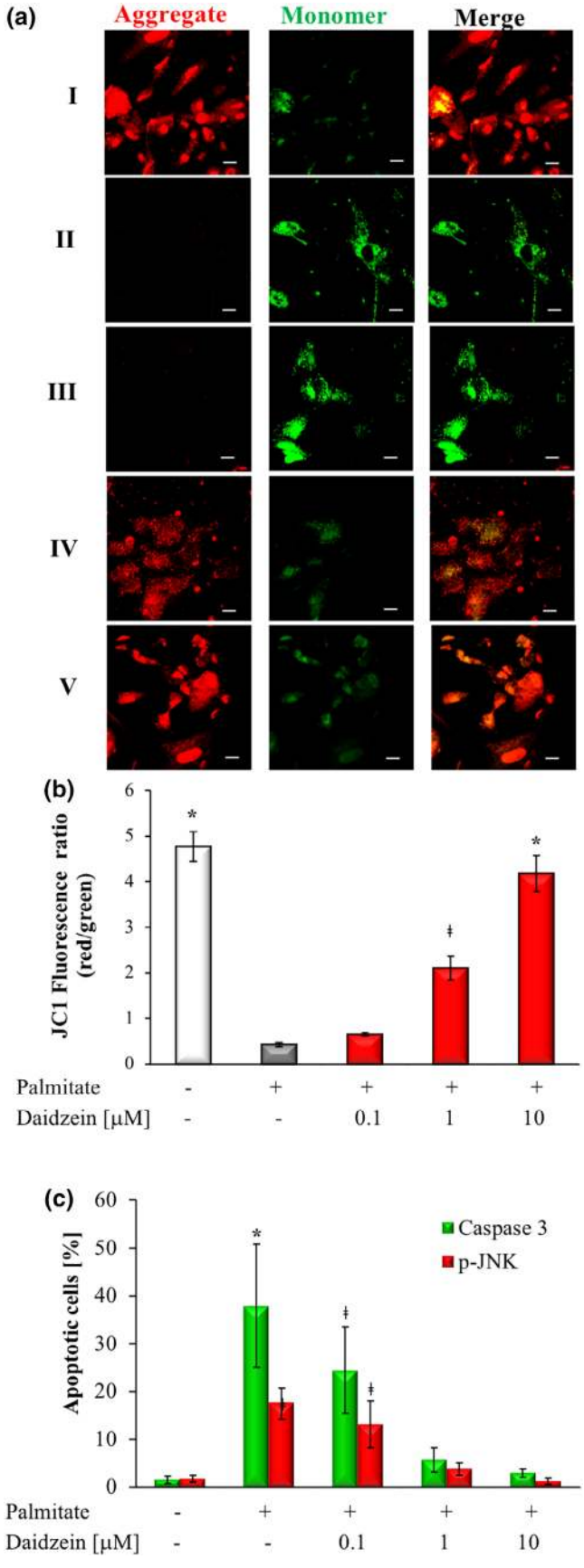


FIGURE 5 Anti-oxidant effect of Daidzein. Daidzein inhibited palmitate-induced ROS generation in neuronal cells. (A) Confocal laser micrographs displaying DCF fluorescence signal in hypothalamic GnRH neurons in basal condition (Control) (I), after palmitate treatment (300 μM) (II) and after co-treatment with palmitate and daidzein 0.1 (III), 1 (IV) and 10 μM (V). Scale bar: 20 μm. (B) Quantitative analysis of DCF fluorescence intensity produced in neuronal cells incubated with various concentrations of daidzein and palmitate. The values expressed as average ± SD are the means of five experiments and data statistically significant were evaluated according to ANOVA followed Bonferroni *t*-test ($p < 0.05$): * versus other treatments; ǂ versus control, palmitate + daidzein 1 μM, palmitate + daidzein 10 μM

indicates mitochondrial damage on the basis of the JC-1 dye mechanism: it enters the cells from the cytosol to the mitochondria and forms aggregates which emit a red fluorescence while in apoptotic cells, due to mitochondrial membrane leakage, JC-1 aggregates go into the cytosol in monomeric form which emits green fluorescence. The red fluorescence signal increased by increasing daidzein concentration as visible in the representative confocal images (Figure 6IV–V) proving that the investigated molecule prevented the mitochondria damage. Quantification of the fluorescence intensity ratio between red/green channels (Figure 6B), resulting from the proportion healthy/damaged mitochondria, reveals how palmitate totally disrupts the mitochondrial membrane integrity and decreases the MMP. The ratio between red/green fluorescence signal increases as an effect of the diadzein exposure, with a rescue activity more evident at the daizein higher concentration. Daidzein prevented MMP collapse exerting a decisive neuroprotection, especially at the concentration of 10 μM the palmitate side effects are totally counterbalanced. Next, we investigated a more specific aspect of apoptotic process through the immunostaining for apoptotic markers by localizing and quantifying the presence of positive cell for Caspase 3 and p-JNK (Figure 6C). After palmitate treatment 38% ± 13% and 18% ± 3% of the cells are positive for Caspase 3 and p-JNK, respectively, acting as pro-apoptotic cell death inducers. In line with the previous evidence, the co-treatment with daidzein reversed the trend and although cells were still under the effect of 300 μM palmitate, the synergistic treatment with daidzein inhibited the palmitate neuro-toxic effects and the related cells death. Indeed, the number of cells positive for the two markers significantly decreased, obtaining at the daidzein highest concentration (10 μM) values of 3% ± 1% and 2% ± 0.7%, for caspase 3 and p-JNK, respectively, which correspond to the same positive cell count observed in the control. Disclosing the anti-apoptotic effects of daidzein was highlighted another action through which the phytoestrogen exerts neuroprotection.



3.5 | Daidzein prevents the palmitate-induced hfHypo cell dysfunction

As previously described, in standard culture conditions hfHypo cells widely express markers of the neuronal lineage, such as β-Tub III and NeuN, as a proof of a proper degree of differentiation. The GnRH phenotype is also confirmed by the expression and cellular distribution of the two specific markers GnRH and KISS1R and by the positive staining for the estrogenic receptors ERβ and GPR30 (Figure 7A). The capacity of daidzein to provide neuroprotection was also investigated by assessing its mediated effect to preserve the cellular structural integrity under the palmitate inflammatory stimulus. Palmitate toxicity induced a consistent reduction in cell number and the complete disruption of the normal structure organization with a drastic reduction in the expression of the neuronal marker β-Tub III and NeuN (Figure 7A). In damaged hfHypo cells, the GnRH phenotype markers, GnRH and KISS1R are weakly expressed. The estrogenic receptors ERβ and GPR30 are also visible only in a small fraction of cells; thus, palmitate evoked its neurotoxicity through a massive neuronal dedifferentiation and impacting the functional integrity of the GnRH secreting cells. A complete protection was offered to the cells by the co-treatment with daidzein 10 μM; the presence of the phytoestrogen totally compensated the palmitate damage. Confocal pictures reveal healthy cells expressing NeuN and with a uniform distribution of the β-Tub III along the unipolar neurite elongation. GnRH peptide, KISS1R and the estrogenic receptors are broadly

FIGURE 6 Anti-apoptotic effect of daidzein. (A) Daidzein protected GnRH neurons against palmitate-induced mitochondrial membrane depolarization. Representative confocal images of neurons displaying JC1 monomers (green) and aggregates (red) in basal condition (Control) (I), after palmitate treatment (II) and after co-treatment with palmitate (300 μM) and daidzein 0.1 (III), 1 (IV), and 10 μM (V). (B) Quantitative analysis of the fluorescence intensity ratio between JC-1 red and green. The values expressed as average ± SD are the means of five experiments and data statistically significant were evaluated according to ANOVA followed Bonferroni *t*-test ($p < 0.05$): * versus other treatments; ǂ versus palmitate and palmitate + daidzein 0.1 μM treatments. (C) Daidzein inhibited palmitate-induced apoptotic markers activation. Quantitative analysis of apoptotic markers Caspase-3 (green bar) and p-JNK (red bar). The values expressed as average ± SD are the means of five experiments and data statistically significant were evaluated according to ANOVA followed Bonferroni *t*-test ($p < .05$): * versus other treatments; ǂ versus control, palmitate + daidzein 1 and 10 μM

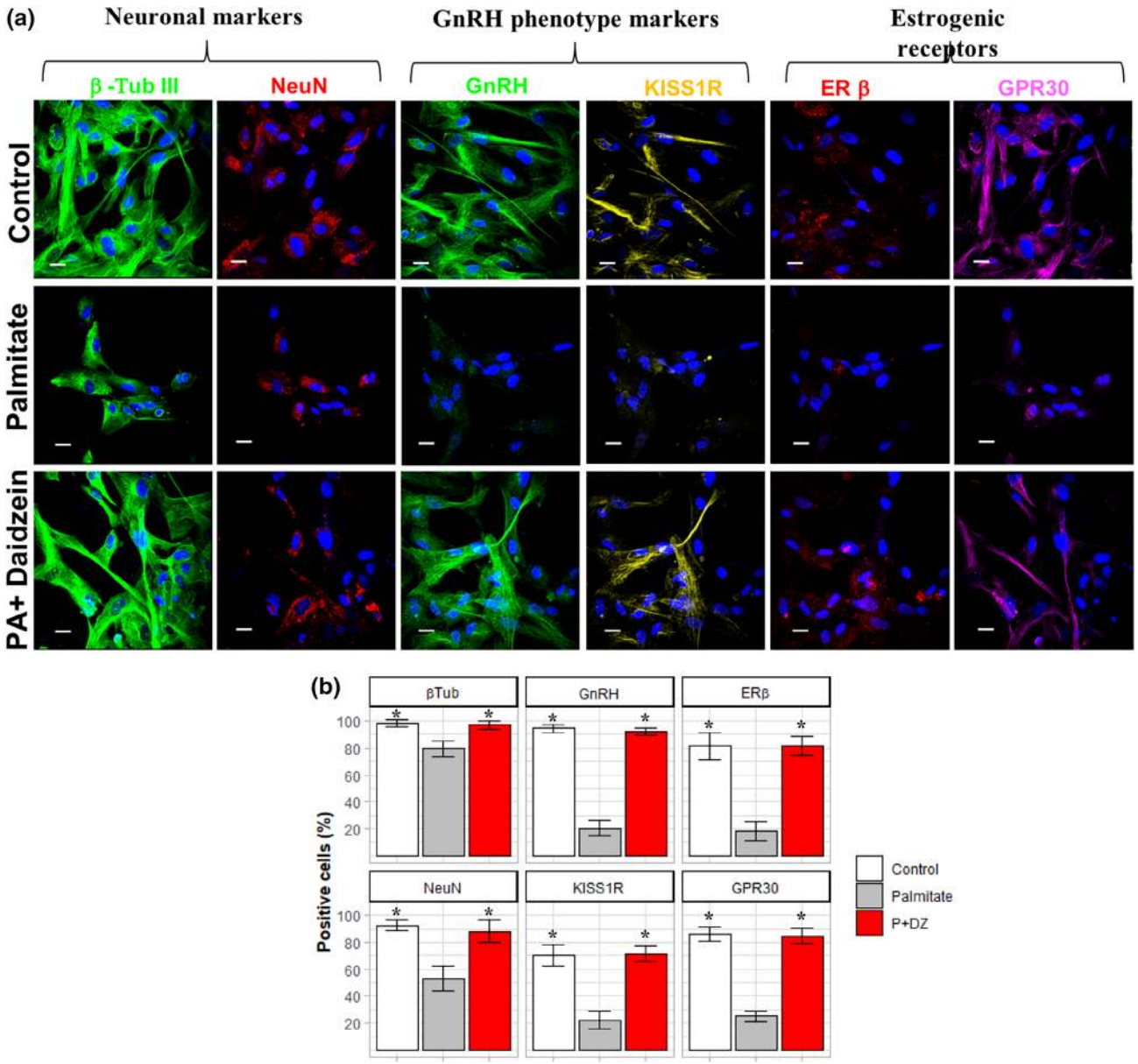


FIGURE 7 Daidzein protected GnRH neurons against palmitate-induced cell damage. (A) Confocal laser micrographs of neuronal cells in PLGA membrane system after different culture conditions: cells without any treatment (Control), Palmitate treatment (Palmitate), and co-treatment with palmitate and daidzein 10 μM (PA+ DZ). Distribution pattern profile of neuronal markers β-Tub III (green), and NeuN (red), GnRH phenotype markers KISS1R (yellow) and GnRH (green), estrogenic receptors ER β (red) and GPR30 (magenta). Nuclei are counterstained with DAPI (blue). Scale bar: 20 μm. (B) Quantitative analysis of positive cells for each marker. Results are expressed as percentage of positive cells over total and are reported as average ± SD of the means of five experiments. Data statistically significant were evaluated according to ANOVA followed Bonferroni *t*-test ($p < 0.05$): * versus palmitate treatment

expressed evidencing that daidzein prevented palmitate toxicity protecting cells from losing the peculiar phenotype markers. The profile expression patterns for each of the above-mentioned markers was also quantified and expressed as the percentage of positive cells over the total number of cells within the investigated image field (Figure 7B). After palmitate treatment, the percentage of cells positive to the neuronal markers β-Tub III and NeuN, is of 80% ± 6% and 53% ± 9%, respectively, which are values significantly lower compared with the control. The number of cells expressing the neuronal lineage proteins increases to 97% ± 3% for β-Tub III and to 88% ± 9% for NeuN when the fatty acid is administered together with daidzein. This trend is kept constant and even emphasized for the other markers, where the percentage fluctuations of positive cells are more evident. In

Figure 7B is clearly visible a drastic downregulation of cells expressing GnRH (20% ± 6%), KISS1R (22% ± 7%), ERβ (18% ± 7%), and GPR30 (25% ± 4%) in response to palmitate insult. In presence of daidzein the percentage of hfHypo positive cells for these specific proteins significantly increases to values of 92% ± 2%, 72% ± 6%, 81% ± 7%, and 84% ± 6%, respectively, in the same range of the control. Daidzein suppresses the induced palmitate cell disfunctions, and the inhibitory palmitate effect over the GnRH phenotype markers and estrogenic receptors expression is not detectable anymore. Thanks to the daidzein-mediated neuroprotection the markers expressions remain within a physiological range as a proof of an appropriate cell functionality.

4 | DISCUSSION

Overnutrition-induced hypothalamic inflammation due to high-fatty acid diet has become a serious public health problem because it is associated with severe consequences, including obesity, diabetes, cardiovascular and neurodegenerative diseases. Thus, many efforts are directed to identify the compounds or molecules that can inhibit or reduce the activation of inflammatory signaling in hypothalamic neurons in order to prevent or treat obesity and related diseases. The challenge is to stop the progressive neurodegeneration by inducing neuroprotection.

The present study provided novel insights on the anti-neuroinflammatory effects of phytoestrogen daidzein, potentially relevant to the understanding of the neuroprotective role of this compound in modulating the inflammatory processes implicated in the evolution of several chronic diseases.

Innovative investigational insights on new and effective therapeutics to treat neurological disorders, and correlated pathological events, require the setting up of a reliable in vitro platform in which the in vivo structure complexity is recapitulated and maintained.

The in vitro reconstruction of GnRH hypothalamic tissue-specific microenvironment, created in this work by using PLGA membrane system, served as model system to test daidzein's ability to stop and reverse the imbalanced neuroinflammatory responses caused by high fat diet.

PLGA membranes, thanks to their morphological, mechanical and physico-chemical properties, enable adhesion, growth and structural and functional neuronal differentiation of the hfHypo-GnRH neurons, by providing a microenvironment well-controlled at molecular level.

In line with a previous study that reports fetal hypothalamus as a rich source of GnRH neurons,[23] hfHypo cells retain their GnRH phenotype in vitro within the PLGA membrane system. Indeed, they displayed a unipolar or bipolar morphology with long processes to connect each other. Immunohistochemical data corroborated the GnRH neuronal phenotype of hfHypo cells, which, besides markers of the neuronal lineage, such as β-Tub III and NeuN, also abundantly express GnRH peptide and the functional kisspeptin receptor, KISS1R, as well as the estrogenic receptor ERβ, and the transmembrane estrogen receptor, GPR30, which has been recently implicated in rapid action of estrogen in GnRH neurons.[27]

The neuronal biohybrid membrane system developed in this work, consisting of the hfHypo-GnRH neurons in culture on the PLGA membrane, reproduces the morpho-functional characteristics of neuronal tissue, and represents, therefore, a valid model-system of hypothalamic tissue. The possibility to set up a realistic biomimetic platform allows to use this system for a breakthrough investigation of the pathogenic mechanisms at the basis of the fatty acids/diet neuroinflammation, and also for the screening of bio-molecules to evaluate their pharmacological potential. With this aim, the PLGA membrane hypothalamic tissue model was used to induce neuroinflammation associated with obesity, in order to investigate the potential anti-neuroinflammatory effect of phytoestrogen daidzein.

To mimic the inflammatory process associated with obesity, cultured cells in the PLGA membrane system were treated with palmitate, a SFA widely present in the diet, whose excessive intake may induce a hypothalamic inflammatory state, as demonstrated by several studies in the literature and confirmed by the present work. The toxic effect of palmitate involves an increase of pro-inflammatory cytokines, elevated oxidative stress, and can result in the death of neuronal cells by apoptosis via a JNK-dependent pathway.[3,25]

We have demonstrated, for the first time in primary cultures of hypothalamic GnRH neurons of human origin, that the proinflammatory events induced by palmitate treatments have involved the increase of inflammatory cytokines, reduction of cell viability, changes in morphology, oxidative stress, and cell death by apoptosis. Moreover, palmitate action also implicates the reduction of specific cellular functions by inhibition of the expression of neuronal markers as well as the GnRH peptide, kisspeptin receptor (KISS1R) and estrogenic receptors (ERβ and GPR30). Then, we evaluated whether daidzein may modulate the deleterious effects triggered by palmitate administration and related to obesity-induced neuroinflammation in primary cultures

of human GnRH neurons by using the developed neural membrane system that mimics the in vivo conditions. In particular, the study characterized the effects of daidzein on palmitate-treated GnRH neurons, with the aim to evaluate the potential capabilities of this phitoestrogen to maintain normal cellular phenotype and protect from oxidative damage, apoptosis, and the deleterious effects of palmitate-induced inflammation on GnRH neuronal functions.

The morphology of neuronal cells reflects their structural and functional integrity, and therefore represents an index of neuronal growth, viability, and differentiation. We observed how palmitate treatment induced a significant decrease of the number of cells and morphological changes of GnRH neurons that assumed a round shape of the cellular body with high neurite loss, which is one of the hallmarks of neuronal injury and neurodegeneration. It is evident, therefore, that the palmitate caused a cellular damage capable of modifying the morphological characteristics typical of such kind of neurons, which are indispensable to accomplish the normal physiological cellular functions.

Daidzein restored the morphological characteristics observed in the control cells, consisting of untreated cells, and furthermore, induced an increase in cell viability in a concentration-dependent manner highlighting the neuroprotective action of daidzein against the toxicity induced by palmitate. These findings are consistent with previous studies that showed that the administration of daidzein caused increased survival of cortical neurons exposed to oxygen and glucose deprivation,[12] significantly promoted the vitality and proliferation of hippocampal neurons and stimulate the neuritogenesis process.[28]

To elucidate the anti-inflammatory mechanism induced by daidzein, further investigations were carried out focusing on the involvement of cytokines production, such as IL-6 and IL-10 that have emerged as key regulators of neuroinflammation. IL-6 is classified as a pro-inflammatory cytokine that may have an anti-inflammatory effect negatively regulating the inflammation of acute phase response. In models of brain injury, infection, LPS injection, or diet-induced obesity, IL-6 is upregulated and modulates inflammation, apoptosis, and oxidative stress. Alternatively, IL-6 is also involved in dampening of the immune responses to promote recovery and healing.[24]

IL-10 is an essential anti-inflammatory cytokine that is released from several cell types, after immune challenge and brain injury, and is responsible for tissue remodeling and restoration of homeostatic conditions. Barabas et al., provided the first evidence that IL-10 directly regulates the functions of GnRH neurons.[29]

Daidzein suppressed palmitate-mediated production of the investigated inflammatory cytokines demonstrating that daidzein had also direct anti-inflammatory properties in addition to its function to prevent cell death. These results are in agreement with a previous report, in which daidzein treatment significantly attenuated the inflammatory IL-6 expression in an in vitro model of adipose inflammation,[13] suppressed IL-10 secretion from LPS treated human dendritic cells,[30] and attenuated cisplatin-induced inflammatory cytokines interleukin 10 (IL-10) in mice with renal damage.[9] To the best of our knowledge, this is the first study showing that daidzein inhibits pro-inflammatory cytokines in human GnRH neurons. Additionally, Ropelle et al., report that recombinant IL-6 requires IL-10 expression to suppress hyperphagia in the hypothalamus of obese animals.[31] IL-6 induces an anti-inflammatory environment by inducing the production of IL-10. Therefore, we can hypothesize that palmitate-induced increase of IL-10 levels may be related to the IL-6 anti-inflammatory response, which involves the increase of IL-10 expression in the hypothalamus, accordingly with Ropelle.[31] In addition, to evaluate the balance between pro- and anti-inflammatory cytokines which controls various physiological processes and the imbalance of which is related to several human diseases, the decrease of IL-6/IL-10 ratio obtained administering daidzein in palmitate-treated cells demonstrates its role in inhibiting the inflammation process.

It is noteworthy that cytokines had an effect on gene expression in GnRH neurons or neuron function, since GnRH neurons express cytokine receptors.[32] Indeed, we previously demonstrated deleterious effects of TNFα on KISS1R signaling and GnRH secretion in the same cellular model.[23] In the present work, we found an effect of palmitate-induced inflammation on cytokines production and phenotype markers, as well as estrogenic receptors. In particular, the palmitate inflammation induced an increase of IL-6 and IL-10 and a decrease of KISS1R, GnRH, ERβ, and GPR30 expression highlighting a relationship between cytokines and markers of phenotype and estrogenic receptors, in accordance with previous studies.[23,32] This effect was completely reversed by daidzein treatment that plays a neuroprotective role in impaired hfHypo cells.

Particular attention was paid to the ability of phytoestrogen daidzein to counteract the negative and toxic effects caused by palmitate on phenotype and function of hypothalamic GnRH neurons. Palmitate treatment caused a very low expression and localization of typical neuronal lineage markers such as β-Tub III and NeuN that leads to the loss of their well-defined neuronal morphology. The SFA exposure determined a specific

inflammatory intracellular signaling characterized by reduced GnRH and KISS1R expression.

This direct inhibitory effect on KISS1R expression observed in our study is consistent with recent reports that demonstrated the association of impairment caused by inflammatory injury with a downregulation of KISS1R in GnRH neuron.[23,33] Our finding supports the idea that the deleterious effects of inflammation on GnRH neurons function, activated by palmitate, are exerted through the decrease of GnRH and KISS1R expression. In line with our expectations, the administration of palmitate was able to decrease not only the number of neuronal cells, but it also negatively affected the phenotype and function of hypothalamic GnRH-secreting neurons.

Daidzein restored the neuronal morphology and the correct GnRH phenotype. The intense distribution of β-Tub III and NeuN markers indicated the neuronal phenotype of primary culture. Daidzein significantly inhibited the adverse effects of palmitate on GnRH expression, accordingly with *in vivo* data that showed how the administration of daidzein determined a marked up-regulation of the levels of mRNA of GnRH in the hypothalamus.[34] Accordingly, daidzein-treated hfHypo cells exhibited the predominant hypothalamic GnRH/KISS1R phenotype, as demonstrated by immuncytochemical analysis. The ability of daidzein in restoring both GnRH and KISS1R expression are extremely relevant by a functional point of view, since kisspeptin is the main physiological regulator of the hypothalamic GnRH system, which governs the reproductive function.

The protective actions of phytoestrogens are mediated by the activation of intracellular nuclear receptor family, ERα and ERβ, which are located in the cytoplasm or on the nuclear membrane. Recent studies have suggested that the transmembrane G protein-coupled receptor 30 (GPR30) may be involved in estrogen anti-inflammatory action. Using human fetal primary cholinergic neurons, it has been recently demonstrated that GPR30 is specifically implicated in mediating neuroprotective effects of estrogens under inflammatory stimulus.[27] Other investigations report that daidzein in the presence of glutamate, used to induce toxicity in primary hippocampal neurons, decreased the level of ERβ without significant changing of the GPR30 protein level.[35] Our results showed, for the first time that daidzein is able to increase the expression levels of both ERβ and GPR30 in the presence of palmitate-induced inflammatory stimulus in hypothalamic GnRH neurons, thus suggesting that these receptors could be involved in the mechanism of action of daidzein in determining neuroprotection against neuroinflammation of obese individuals. In support of this hypothesis, scientific data showed that daidzein, thanks to its structural homology with 17β-estradiol, is able to bind to the classical estrogenic receptor ERβ in hippocampal neurons, stimulating the growth of their axons[36]; while more recent data suggest that its main metabolite equol is able to inhibit neuroinflammation induced by LPS in astrocytes by pathway mediated by the GPR30.[37]

SFA induce mitochondrial damage and oxidative stress that are accompanied by excessive production and/or insufficient removal of ROS. In general, isoflavones are known to be antioxidant, and, indeed, daidzein suppressed ROS production in microglia during LPS-induced neuroinflammation,[8] and also in mouse models of cerebral ischemia reduced oxidative stress caused by increased activity of the enzyme superoxide dismutase.[38] The neuroprotection effect of daidzein could be due to its chemical structure that allows to play a role of scavenger of free radicals by hydrogen atom donation, protecting in this way cells from oxidative damage. Herein, we have demonstrated that the treatment with daidzein avoids palmitate-induced ROS production that is responsible for cell impairment. These results clearly indicate and thus confirm the antioxidant properties of such phytoestrogen.

It is known that the neuroinflammation associated with obesity is characterized also by the apoptotic process activation. Excessive ROS, induced by palmitate, can lead to mitochondrial dysfunctions with loss of the mitochondrial membrane potential (MMP), which is widely considered as an early intracellular event of the apoptotic cascade. Palmitate caused a drastic depolarization of MMP and the treatment with daidzein had a concentration-dependent protective effect on mitochondria. Therefore, our results demonstrated that daidzein can prevent the palmitate-induced collapse of MMP, suggesting a strong protective effect against palmitate-induced mitochondrial dysfunction. Thus, daidzein neuroprotective action could be related to the recovery of mitochondrial bioenergetics and functions, and the capacity to inhibit the apoptotic process triggered by palmitate, suggesting therefore its anti-apoptotic effect.

Several molecular studies provided evidences that inflammatory responses are mediated by JNK activation.[39] Recently, Tse et al.[25] demonstrated that activation of JNK is required for palmitate-induced inflammation, specifically, other authors report that SFA palmitate, may induce neuroinflammation through pathways involving the activation of JNK and caspase 3.[2] Moreover, daidzein inhibited secretion of pro-inflammatory cytokines in adipocyte-macrophage co-cultures via multiple pathways in which JNK activation is involved.[13]

Within this scenario, we further explored the daidzein anti-apoptotic effect by investigating the ability of

this phytoestrogen in preventing palmitate-induced activation of JNK and Caspase-3, since they are implicated in the sequence of events leading to apoptosis. Indeed, JNK represents the upstream sensor protein, which is activated following a condition of oxidative stress due to palmitate treatment, and caspase 3 represents the downstream apoptotic effector molecule. To have a general overview at upstream and downstream of JNK activation, we have evaluated the capacity of daidzein to inhibit ROS production, since it acts as potent inducer of JNK, and one of the antiapoptotic mechanisms is to remove the ROS to prevent JNK activation.[40] JNK is also involved in other apoptotic signal activation as the downstream mitochondrial/caspase-9 pathway whose activity converges on the activation of caspase-3, which is then a downstream effector of JNK pathway.[41] Importantly, the treatment with daidzein blocked JNK phosphorylation and caspase-3 cleavage and, thus, prevented the deleterious effects of palmitate by regulating signaling pathways within neurons and inhibiting the onset of the apoptotic process. The study also analyzed the loss of mitochondrial membrane potential that is considered as an early indicator of apoptosis. Therefore, we sought for representative events that occur in the apoptotic cascade, with the final goal to provide evidence about daidzein's anti-apoptotic effect, to gain insight into the mechanism underlying the daidzein-induced neuroprotection.

Overall, our data demonstrated that cell death, morphological changes, interleukins and ROS production, as well as apoptotic state, and impaired functionality evoked by palmitate-activated neurons, were significantly inhibited in a dose-dependent manner by daidzein co-treatment. The putative mechanism of the neuroprotetctive effect of daidzein against palmitate-induced hypothalamic inflammation is summarized in Figure 8.

On the basis of the collected data we can suppose that daidzein, thanks to its chemical structure, which includes a phenolic ring, can directly bind to the estrogen receptors and regulate the hypothalamic cellular functions, coherently with other studies.[42] Overall, the present study highlights the therapeutic potential of daidzein in

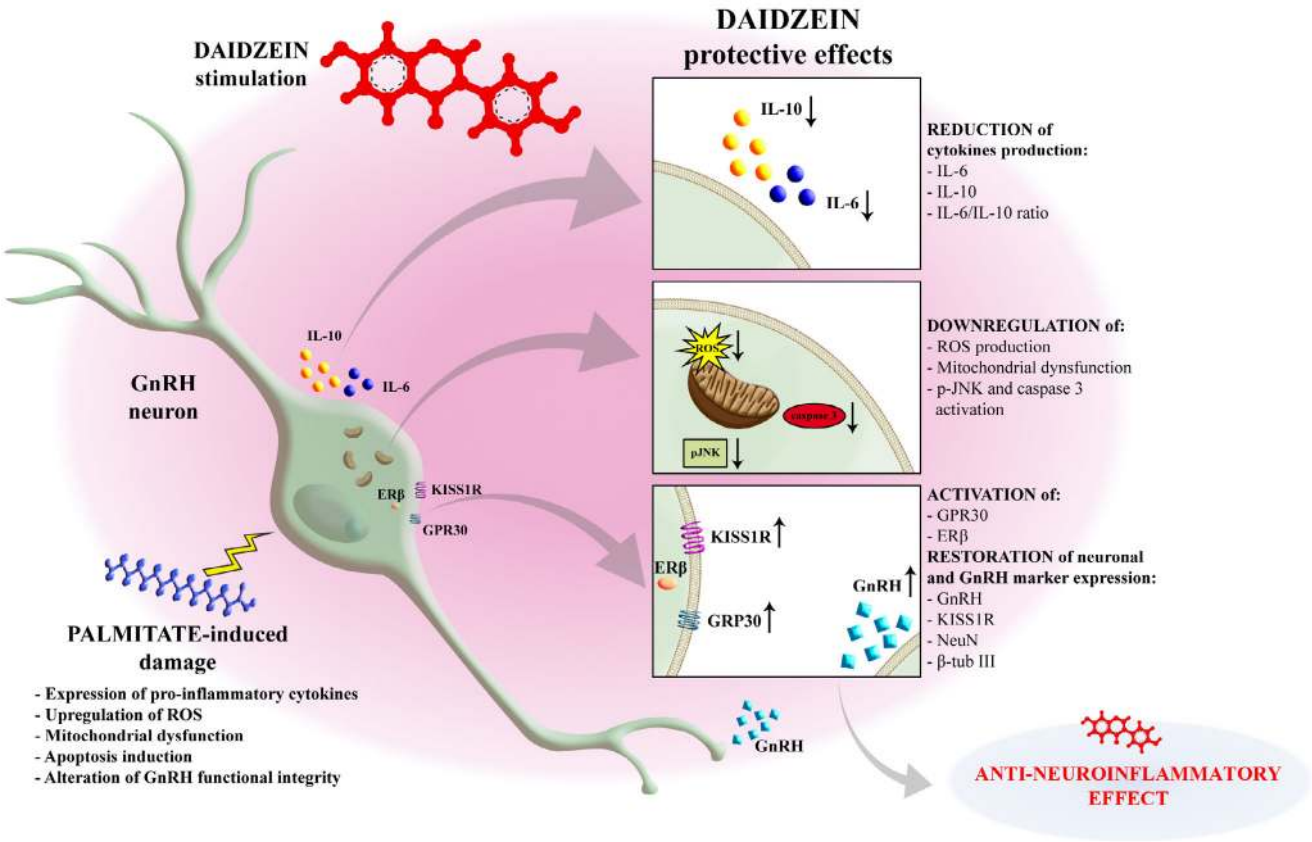


FIGURE 8 Anti-neuroinflammatory effect of daidzein in GnRH neurons. Daidzein was able to counteract the detrimental effects of palmitate by eliciting an increased viability and diminishing ROS and interleukins production. Daidzein displayed anti-apoptotic action by downregulating mitochondrial dysfunction and p-JNK and caspase-3 activation. Importantly, daidzein upregulated markers of neuronal linage and GnRH phenotype and also mediated the activation of ERβ and GPR30 receptors in palmitate-treated cells

the prevention of neuroinflammation caused by high-fat diets.

5 | CONCLUSIONS

This study provides novel insight into the anti-neuroinflammatory effects of daidzein on hfHypo cells. PLGA membrane system enhanced the creation of functional hypothalamic neuronal tissue analogue that was successfully employed as a valuable tool for testing the neuroprotective effect of daidzein against high fat diet-induced inflammation.

The molecular mechanism of daidzein neuroprotection was mediated through downregulation of palmitate-induced cell impairment. Specifically, the phytoestrogen not only inhibited palmitate-induced neuronal death and pro-inflammatory cytokines secretion, but also protected from neuroinflammatory injury through downregulation of ROS production, mitochondrial dysfunction and p-JNK and caspase-3 activation, thus demonstrating both antioxidant and anti-apoptotic action. Furthermore, daidzein was able to suppress the inhibitory effect of palmitate on the expression of specific markers of neuronal linage and GnRH phenotype, suggesting its role in restoring the correct neuronal morphology and functionality of hfHypo GnRH cells. In addition, its protective action was also mediated by the activation of ERβ and GPR30 receptors.

Our data highlight the therapeutic potential of daidzein in counteracting neuroinflammation caused by high-fat diets, and therefore, in the prevention of the related diseases. Since GnRH neurons play a key role in controlling the hormonal network crucial for sustaining the reproductive function, our findings could advance the understanding not only of the mechanisms underlying daidzein neuroprotective effect, but also of the close relationship between obesity-related neuroinflammation/neurodegeneration and reproductive function.

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CONFLICT OF INTEREST

All authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

Sabrina Morelli: conceptualized, designed and carried out experiments, analyzed results and wrote the manuscript. Antonella Piscioneri: prepared membrane system, performed cell culture experiments, immunostaining, contributed to analyzing and writing results. Giulia Guarnieri: contributed to cell cultures; Annamaria Morelli: contributed to the interpretation of the results and revised the manuscript. Enrico Drioli: revised the manuscript. Loredana De Bartolo: designed experiments, analyzed results and revised manuscript. All authors read and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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