Increasing protective activity of genistein by loading into transfersomes: A new potential adjuvant in the oxidative stress-related neurodegenerative diseases?

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Abstract

Background: Genistein is a soy-derived isoflavone and phytoestrogen with antioxidant and neuroprotective activity. Genistein has intrinsically low oral bioavailability that affects its dose-response activities.
Purpose: Nanotechnologies were used to obtain the delivery of genistein to the brain: lipid-based nanovesicles, transfersomes, loaded with the phytoestrogen were developed as potential therapeutic or preventive strategy against neurodegenerative diseases by intranasal administration.
Methods: Phosphatidylcholine from soybean and different edge activators were used to prepare transfersomes. The effect of selected nanovesicles on the oxidative damage was studied in PC12 cell line.
Results: Suitable nanovesicles as carrier of genistein were obtained; their composition affects deformability, drug permeation behavior and cytotoxicity. In particular, the formulation containing Span 80, GEN-TF2, showed efficiency of internalization into the cell and it was able to attenuate ROS formation and to reduce the amount of apoptotic cells generated by H_2O_2 treatment compared to genistein.
Conclusion: GEN-TF2 was able to reduce the oxidative damage suggesting a possible antioxidant role of this drug delivery system. These obtained data confer to GEN-TF2 a potential antioxidant activity and then it could be used as adjuvant therapy in oxidative stress-related neurodegenerative diseases.

Keywords

Genistein; Vesicular carrier; antioxidant; oxidative stress; PC12 cell; neurodegenerative diseases

Abbreviations: AD, Alzheimer's disease; BBB, Blood-brain barrier; CNS, Central nervous system; DMEM/F12, Dulbecco's modified Eagle's medium; FBS, Fetal Bovine Serum; GEN, Genistein; GEN-TFs, GEN-loaded transfersomes; H_2O_2, Hydrogen peroxide; HLB value, hydrophilic-lipophilic balance; HPLC, High performance liquid chromatography; HS, Horse Serum; LDH, Lactated hydrogenase; MTT, 3-(4, 5-dimethyl- thiazol-2-yl)-2, 5, diphenyl tetrazolium bromide; Na-DOC, Sodium deoxycholate; NDs, Neurodegenerative diseases; PBS, Phosphate-buffered saline; PC12 cells, Rat pheochromocytoma-derived cell line; PD, Parkinson's disease; PDI, Polydispersity index; ROS, Reactive oxygen species; SD, Standard Deviation; TFs, Unloaded transfersomes;TF1 and GEN-TF1, Unloaded and loaded transfersomes containing Tween 80; TF2 and GEN-TF2, Unloaded and loaded transfersomes containing Span 80; TF3 and GEN-TF3, Unloaded and loaded transfersomes containing sodium deoxycholate; TF4 and GEN-TF4, Unloaded and loaded transfersomes containing Span 20.

Introduction

The role of oxidative stress in the pathogenesis of neurodegenerative diseases has been well demonstrated in many preclinical and clinical studies. Oxidative stress is a condition of imbalance between production of reactive oxygen species (ROS) and antioxidant defenses, resulting in excessive accumulation of ROS. Oxidative stress may induce cellular membrane damage from lipid peroxidation, changes in protein structure and function, structural damage to DNA, and mitochondrial dysfunction, which accelerate the aging process and the development of neurodegenerative disorders (Kim et al, 2015).

Neurodegenerative diseases (NDs) are characterized by progressive damage in neural cells and neuronal loss, which lead to compromised motor and/or cognitive function. Neuron cells are particularly vulnerable to oxidative damage because of their high polyunsaturated fatty acid content in membranes, high oxygen consumption, and weak antioxidant defense (Rego and Oliveira, 2003).

Therefore, the development of antioxidant strategies may help to retard or minimize the oxidative damage. Polyphenolic antioxidants are among the most widely employed natural compounds to protect cellular components. Among polyphenolic compounds, flavonoids display neuroprotective properties in preclinical models of Alzheimer's disease (AD), and Parkinson's disease (PD).

Genistein (GEN) is an isoflavonoid found in several leguminosae, particularly in the soybean, with numerous prophylactic health effects. Genistein exhibits potent antioxidant property (Mazumder and Hongsprabhas, 2016), and neuroprotective effect by modulating cholinergic and dopaminergic functions (Soni et al., 2016), by attenuating the formation of Aβ plaques (Liao et al., 2013), and by reducing Aβ induced oxidative damage (Sadhukhan et al., 2018). Several pre-clinical studies on GEN indicates its promising therapeutic activity against the pathogenesis of AD. As phytoestrogen, its neuroprotective activity is also due to its ability to modulate the inflammatory responses (Du et al., 2018). However, the clinical applications of GEN, as well as other polyphenolic compounds, are restricted by their extensive metabolism, low aqueous solubility, low oral bioavailability (Yang et al., 2012), but it can cross the blood-brain barrier (BBB) (Du et al., 2018). Furthermore, the therapeutic effects of antioxidants on neurodegenerative disorders are poor; this is potentially due to inadequate dosage or timing of administration, or the unsuitable duration of therapy (Kim et al, 2015; Liu Z. et al., 2017).

Nanotechnology could potentially revolutionize treatment of NDs by affording targeted drug delivery and enhancing the bioavailability and/or efficacy of various drugs and other bioactive agents used in NDs (Sandhir et al., 2015). Among lipid-based nanovesicles, liposomes and their modification, such as transfersomes, ethosomes, and phytosomes, which differ from liposome only for composition, were extensively proposed as suitable carriers for improvement of NDs treatment (Mancini et al., 2018). They are spherical bilayered phospholipid vesicles enclosing an aqueous inner core and, thus, are considered biocompatible and non-toxic (Wen et al., 2017). Specifically, transfersomes are ultra-deformable vesicles due to the presence of a surfactant (or edge activator) combined to the phospholipid. This important feature renders transfersomes suitable formulations for the transdermal administration of different drugs, as well as for the transmucosal drug delivery, i.e. through nasal mucosa, for both systemic and neuronal targets (Salama et al., 2012; Aboud et al., 2015).

Intranasal delivery is a noninvasive method to bypass the BBB and directly deliver drugs to the brain due to the unique anatomic connections provided by the olfactory and trigeminal nerves that connect the nasal mucosa and the CNS (Pardeshi and Belgamwar, 2013). However, due to the mucociliary clearance and the poor mucosal permeability of several drugs, an important requirement for this route is the optimization of the drug absorption process, through the development of appropriate delivery systems (Gavini et al., 2009; Gavini et al., 2013; Rassu et al., 2015; Yalcin et al., 2016).

From this background, the aim of this study was to obtain the delivery of GEN to the CNS by using nanotechnologies: therefore, transfersomes were developed to properly encapsulate and transport GEN, as potential therapeutic or preventive strategy against NDs. Further goal was to explore the effect of the GEN-loaded transfersomes against oxidative damages in PC12 cell line compared with GEN.

Materials and Methods

Materials

Synthetic genistein (98% pure by HPLC; PubChem CID: 5280961) (GEN) was purchased from Farmalabor (Milan, Italy). Phosphatidylcholine from soybean (Lipoid S100) was a kind gift from Lipoid GmbH (Ludwigshafen, Germany). Tween 80 and sodium deoxycholate (Na-DOC) (≥ 97%) were purchased from Sigma-Aldrich (Milan, Italy); Span 80 and Span 20 were obtained from Merck (Darmstadt, Germany). Acetic acid was purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile and methanol of chromatographic grade were purchased from Merck (Darmstadt, Germany). HPLC grade water was obtained using a Milli-Q system from Millipore (Bedford, CA, USA). All other chemicals and reagents were of analytical grade. Dulbecco's modified Eagle's medium (DMEM/F12, HEPES, no phenol red), streptomycin/penicillin, horse serum (HS) and fetal bovine serum (FBS) were acquired from Life Technologies. Hydrogen peroxide (H_2O_2, 30%), phosphate-buffered saline (PBS, 0.2 µm filtered, adjusted to pH 7.4) solution, trypan blue (0.4%), 3-(4,5-Dimethyl-thiazol-2-yl)-2,5,diphenyltetrazoliumbromide (MTT, 97.5%), propidium iodide solution and RNase A were purchased from Sigma-Aldrich. ROS-ID® Total ROS Detection and LDH-Cytotoxicity Assay Kit II were acquired from Enzo Life Sciences and BioVision respectively. PE Annexin V Apoptosis Detection Kit was purchased from BD Pharmingen and fluorescein from Fluka Analytical.

Preparation of transfersomes

Transfersomes, both unloaded (TFs) and GEN-loaded (GEN-TFs), were prepared by a slightly modified rotary evaporation-sonication method (Kong et al., 2014). The mixed lipid, consisting of the phosphatidylcholine and the edge activator in the selected ratio (Table 1) was dissolved in 100 ml of ethanol and placed in a dry, round bottom flask. Based on preliminary studies, the appropriate amount of GEN was added to this mixture in case of, GEN-TFs (Table 1). The organic solvent was removed under vacuum (Rotavapor RE111, Büchi Labortechnik

AG, Flawil, Switzerland) at 40°C for 20 min and the thin film obtained was hydrated with 50 ml of ultra-pure water by rotation for 30 min at 30°C. The resulting vesicles were swollen for 1h at room temperature to obtain multilamellar vesicles (MLV). Afterwards, they were sonicated by a probe sonicator at 20 KHz for 10 min, to reduce vesicle size. Four type of TFs and GEN-TFs were obtained, according to the different edge activators proposed (Table 1).

Fluorescent GEN-TF2 were also prepared for cell culture studies. Fluorescein was added to the mixture of phospholipid, edge activator and ethanol, by substituting the exact amount of GEN, according to the GEN-TF2 composition. After fluorescein was completely dissolved, the organic solvent was removed under vacuum, following the preparation method above described.

Characterization of transfersomes

Physical characterization of transfersomes

Vesicle size and size distribution measurements of TFs and GEN-TFs were performed by photo correlation spectroscopy, using a Coulter submicron particle sizer N5 (Beckman- Coulter Inc. Miami, FL, USA). Measurements were done in terms of mean diameter of the vesicles and their dimensional homogeneity, expressed as PDI. Before each determination, all samples were properly diluted with bidistilled water, previously filtered through nylon membranes (0.22 µm pore size). Samples were analyzed three times (n=3) and results are expressed as mean ± standard deviation (SD).

Zeta potential were determined by Photocorrelation Spectroscopy using a 90 Plus instrument (Brookhaven, NY, USA) at a scattering angle of 90° and a temperature of 25°C. Transfersome suspensions were diluted in deionized filtered water before measurement. Samples were placed in the electrophoretic cell, where an electric field of approximately 15 V/cm was applied. Each measured value was the average of ten readouts.

The morphology of transfersomes was evaluated by Transmission Electron Microscopy (TEM) (supplementary information).The GEN loading in the GEN-TFs, the storage stability and relative deformability of the TFs and GEN-TFs were determined (supplementary information).

In vitro *permeation studies*

In vitro permeation experiments were carried out on GEN-TFs, to test the capability of GEN-TFs to enhance drug permeation through membranes simulating biological conditions. The test was done using a modified Franz diffusion system incorporating three in-line, flow-through diffusion cells, as previously reported (Gavini et al., 2011). Regenerated cellulose membranes (pore size 0.22 µm, saturated with octanol), were employed as lipophilic layer to mimic nervous cells (Rassu et al., 2015). The receptor compartment was filled with 50 ml of phosphate buffer pH 6.5 at 37°C, containing sodium lauryl sulphate 0.2% w/v. An amount of formulations equivalent to 1.25 ml was uniformly distributed above each membrane. Then, 0.5 ml of acceptor fluid were taken at predetermined time intervals (15, 30, 60, 120, 180 min) and analyzed by HPLC, after dilution with methanol (1:1 v/v).The results were plotted as cumulative drug permeated versus time (n=3±SD).

In vitro *cytotoxicity studies*

Cell culture and drug treatments

PC12 cells, rat pheochromocytoma-derived cell line (ATCCCRL-1721), are a suitable dopaminergic neuronal model. PC12 cells, during their exponential phase growth, were maintained in atmosphere of 5% CO_2/95% humidified air at 37°C in 60 mm plastic culture plates in DMEM/F12 supplemented with 10% HS, 5% FBS and 1% of penicillin/streptomycin. To perform the experiments the cell culture was used during passage numbers 12 until 25. To evaluate GEN-TFs formulations effects cells were treated with GEN (30 μM or 50 μM) or GEN-TF1, -TF2, -TF3, -TF4 (30 μM and 50 μM) either alone or in association with H_2O_2 (75 μM) for 24 h. For each experiments 1×10^5 cells/mL/well were seeded in 24-well plates. All experiments were done in triplicate.

MTT and Trypan Blue assay

After 24h of treatments exposition, the cell viability was assessed by the MTT assay in which, viable cells convert the soluble dye MTT to insoluble (in aqueous media) blue formazan crystals. In brief, 1 mg of MTT (200 μl of a 5 mg/ml stock solution in PBS) was added per milliliter of medium in the culture plate and incubated at 37°C for 4h. The MTT was removed, and the cells were rinsed with PBS and centrifuged at 4,000 rpm for 15 min. Thereafter, the supernatant was discarded, and the pellet was dissolved in 2 ml of isopropanol; after centrifugation at 4,000 rpm for 5 min, the absorbance value was detected at 578 nm employing a Bauty Diagnostic Microplate Reader. Before each experiments, a calibration standard curve was performed. In the same experimental conditions the cell viability was also evaluated with Trypan Blue (0.4%) exclusion assay.

LDH assay

The effect of GEN-TF2 (30 μM) on LDH content in H_2O_2 -treated PC12 cells was performed after 24h of treatments. Cellular LDH release was measured using the LDH-Cytotoxicity Assay Kit II (BioVision) as described in the user's manual instructions.

In vitro cellular uptake

PC12 cells seeded in 6-well plates (1.5 x 10^6 cells/well) were exposed to fluorescein-labelled GEN-TF2 formulation (30 μM and 50 μM). After 24h incubation, the cells were washed three times with PBS and suspended in DMEM/F12 0.25% w/w of trypan blue. The internalization of formulation into PC12 cells was assessed by FACSCANTO flow cytometer (Becton &Dickinson, USA) and the data obtain analyzed by DIVA 6.3 software (BD Bioscience).

Total ROS detection

The intracellular ROS content was evaluated in hydrogen peroxide-exposed PC12 cells (seeded in 6-well plates at 1.5 X 10^6 concentration) treated with GEN-TF2 (30 μM). After 24h administration, the cells were rinsed one

time with 1X Wash Buffer and then incubated with the ROS Detection Solution (Oxidative Stress Detection Reagent, carboxy-H2DCFDA, in 1X Wash Buffer) for 30 min in dark at 37°C. The intracellular production of ROS was detected by flow cytometer (Becton & Dickinson, USA) at the wavelength Ex/Em: 490/525 nm.

Cell cycle

After 24h of GEN-TF2 treatment (30 µM) in presence of H_2O_2, PC12 cells (seeded in 6-well plates at 1.5 X 10^6 concentration) were fixed in cold 70% ethanol, treated with RNase A, and stained with propidium iodide. Cells were analyzed using a FACSCANTO cytometer.

Apoptosis assessment

PC12 cells seeded in 6-well plate at 1.5 x 106 concentration were treated with GEN-TF2 (30 µM) alone or in association with H_2O_2. After 24h incubation PC12 cells were washed with Annexin V binding buffer. The apoptosis was evaluated using Annexin V-PE Apoptosis Detection Kit (BD pharmingen): in detail, cells were incubated with Annexin V-PE/7AADfor 15 minutes in dark at room temperature (20–25 °C).Cells were analyzed using a FACSCANTO cytometer.

Statistical analysis

All experiments are expressed as mean values with 95% confidence intervals and, statistical significance between control and experimental groups was evaluated as significant when two-tailed P values were <0.05. All analysis were evaluated by One-Way ANOVA analysis of variance test using Graph-Pad Prism 5.0 software (GraphPad Software, Inc, San Diego, CA, USA).

Results

Preparation and characterization of transfersomes

Rotary evaporation technique is suitable for obtaining nanovesicles as carrier of genistein. After solvent evaporation, the formed lipid layer is easily hydrated except in case of TF2 and GEN-TF2. Results of vesicle size and PDI are shown in Table 2. Formulations show different mean diameters, based on the nature of the edge activators. TF2 and TF4, as well as GEN-TF2 and GEN-TF4, display a similar behavior, with significantly higher mean diameters than formulations containing hydrophilic edge activators (P<0.05). TF3 and GEN-TF3 have absolutely the smallest size. A significant reduction of the vesicle size occurs for GEN-TF2 and GEN-TF3 (P<0.05), while it is not observed a marked change of the average size after GEN loading for TF1 and TF4 (P>0.05). The PDI values shown (0.3-0.4) indicate a quite narrow and unimodal distribution for almost all TFs,

except TF3 which always demonstrates to be an inhomogeneous formulation, despite the reduced size. The drug loading seems to slightly increase the homogeneity of GEN-TF1, -2 and -4, but without statistical differences (P>0.05).

All transfersomes exhibit the negative zeta potential values; TFs values are in the range of −13.44 mV to −26.15 mV owing to the net charge of the lipid content in the formulations. GEN loading decreases the negative charge of vesicle surface (from - 26.15 ± 2.50 mV of TF2 to - 8.34 ± 1.34 mV of GEN-TF2, reported as example). GEN loading affects the morphology of transfersomes (supplementary information).

The GEN content measured is very similar to the theoretical one for all formulations, except for GEN-TF2 (P<0.05), (Table 2 and supplementary information).

Transfersomes can be considered quite stable (supplementary information), without any precipitation or separation phenomena, and even though an aggregation of the vesicles occurs, mainly after 3 months, the average size remained around 200 nm. Furthermore, storage conditions at 2-8°C are preferred.

Table 2 also lists the relative deformability measured, for both TFs and GEN-TFs. TF2 and TF4 are not able to be extruded through the membrane while TF1 and TF3 show an elasticity of the bilayer than the lipophilic counterparts. The GEN loading significantly affects the relative deformability of TF1 (P<0.05) (supplementary information). Formulations exhibit different drug release profiles according to their different composition and properties (Fig. 1). No GEN permeation is observed for GEN-TF2 and GEN-TF4, being statistically different from all other formulations (P<0.05). On the other hand, GEN-TF1 demonstrates a fast and almost complete GEN permeation after 15 min, which stabilizes with a plateau over the time. The GEN-TF3 has a slow and modulated release during the time of the experiment compared to the other GEN-TFs.

Cytotoxicity of GEN-TFs

MTT and Trypan Blue assay

MTT assay was used to evaluate the eventual toxicity of GEN-TFs in PC12 cells. As shown in Fig. 2, GEN-TF1, -TF2, -TF4 did not induce significant changes on PC12 cell viability at the concentration of 30 μM (98%, 99% and 97% respectively) in comparison with control group. On the other hand, the GEN-TF3 resulted cytotoxic even at lower concentrations (data not shown). At the concentration of 50 μM, all the GEN-TFs exhibited significant decreases of the cell viability.

Subsequently the effect of the different GEN-TFs at 30 μM, except for GEN-TF3, was evaluated on PC12 cells exposed to hydrogen peroxide (75 μM). As illustrated in Fig. 3a (MTT) and 3b (Trypan Blue) GEN 30 μM and GEN- TF4 did not show protective effect when used with H_2O_2 75 μM. GEN-TF1 and GEN-TF2 protected the PC12 cells exposed to H_2O_2 restoring the cell viability to 76% and 81% respectively in comparison with control group. On the basis of the experimental data, the following experiments were carried out using GEN-TF2.

LDH assay

On the basis of the previous results, the LDH assay was performed to further evaluate the GEN-TFs formulations protective effect. In presence of hydrogen peroxide, LDH levels were increased by 44% in

comparison with control (Fig. 4). When the cells were pre-treated with GEN-TF2, the LDH release was reduced by 20% compared to H_2O_2 (Rodríguez-Huamán et al., 2017).

GEN-TF2 cellular uptake

Flow cytometry with the fluorescent GEN-TF2 30 μM formulation has been used to evaluate its uptake in PC12 cells. The amount of internalized GEN-TF2 was represented as percentage of the fluorescent cells. As showed in Fig. 5, the uptake of GEN-TF2 (37%) was higher than GEN alone (7%) at the same concentration. The internalization of GEN-TF2 formulation was higher by increasing the drug dose (Pinna et al., 2015).

Studies of protective effects of GEN-TF2

Total ROS detection

In order to test the antioxidant properties of GEN-TF2, an assay for intracellular ROS detection was performed by flow cytometer. The ROS amount was proportional to the fluorescence intensity. As evident in Fig. 6, the pro-oxidant effect of hydrogen peroxide (37%) was significantly reduced in presence of GEN-TF2 30 μM (6%). GEN was not able to reduce the oxidative damage; on the contrary, it increased the oxidative stress when administered with H_2O_2.

Cell cycle

A further analysis was used to investigate the GEN-TF2 properties. The cell cycle study was performed by flow cytometer. As shown in Fig. 7, no differences were found inG0/G1 and S cell cycle phases among control and GENs groups. The G2/M phase was increased only for the cells treated with H_2O_2 alone (18%) and in addition to GEN (30%) compared to control (10%). In presence of GEN-TF2, the G2/M phase was restored at values comparable to control (12%) (Wang et al., 2016).

Apoptosis assessment

Flow cytometry analysis (Fig. 8) was performed to investigate a possible anti-apoptotic effect of GEN-TF2. The total apoptosis induced by H_2O_2 (40% vs control group) was significantly reduced in cells treated with GEN-TF2 (9% vs control group). The GEN-TF2 formulation restored apoptosis level to control group values (7%). A lower anti-apoptotic effect was exhibited by GEN not loaded (18%).

In support to apoptosis data, the Fig. 9 showed the nuclear staining (Hoecst 33342) of PC12 cells exposed to hydrogen peroxide, clearly reduced in presence of GEN-TF2 (panel C), restoring a nuclear integrity comparable to control group (panel A).

Discussion

Preparation of transfersomes with the rotary evaporation method can be considered a time-saving and efficient

technique, compared to others (El Zaafarany et al., 2010). After the evaporation of the solvent, the formation of a thin lipid film with a large surface area is ensured. This favors an optimal hydration step and suitable formation of the vesicles. However, in the case of TF2 and GEN-TF2, the thin film seems to be more difficult to hydrate than the others, probably due to the highest lipophilicity of Span 80 (HLB value 4.3). As concerning the dimensional properties of transfersomes prepared, they depend on the nature of the edge activators: formulations containing hydrophilic edge activators show lower mean diameters compared to TF2 and TF4. It is interesting to note that TF3 and GEN-TF3 have absolutely the smallest size: despite Na-DOC is a hydrophilic edge activator as Tween 80, it differs from for its ionic nature that might explain this result in comparison with TF1 and GEN-TF1 (Aboud et al., 2015). Although transfersomes can be considered quite stable storage conditions at 2-8°C are preferred, since refrigerated vesicles were found to be reasonably stable in terms of aggregation and fusion (Ghanbarzadeh and Arami, 2013).

The GEN loading decreases the negative charge of vesicle surface. Surface charge plays an important role in cellular uptake: literature data show, indeed, that in non-phagocytic cells (such as PC12 cells) a low negative charge improves the nanoparticles internalization due to the weakened electrostatic repulsion forces between the particles and the cell membranes (He et al., 2010).

The ability to permeate cell membranes is an important feature, worthy of evaluation, of a nanosized delivery system. GEN-TFs show different behaviour, based on the nature of the edge activators: GEN-TF2 and GEN-TF4, indeed, do not release GEN. The combination of two aspects can be hypothesized; first, GEN-TF2 and GEN-TF4 do not release GEN due to, perhaps, the affinity of GEN to vesicles is higher than hydrophilic edge activators. Furthermore, according to the scarce deformability of GEN-TF2, and GEN-TF4, they are not able to cross the lipophilic barrier; instead, they preferentially accumulate within the membrane pores. This fact allows to hypothesize that these formulations have an high affinity for the double-phospholipid layer of neuronal cells, thereby this might affect the mechanism of the nose-to-brain drug transport (Rassu et al., 2015). On the contrary, rapid GEN permeation occurs when GEN-TF1 is tested. The GEN-TF3 curve offers an interesting view, since it has a slow and modulated GEN release despite its reduced size. This can be attributed to the edge activator nature: Na-DOC is the only that have a steroidal structure and can interact with lecithin in a different way, perhaps limiting the GEN release over the time. The permeation behavior of drug could be also due to small fractions of molecules dissolved in the solvent. Solubilizing agents like liposomes, micelles and hydrophilic polymers increase permeability of a poorly soluble drug because of induce the increase of apparent solubility of drugs (di Cagno and Luppi, 2013). In this case, the concentration of molecularly dissolved drug which could freely diffuse through the membrane, could be affected by the kind of edge activator employed.

The *in vitro* cytotoxicity of GEN-TFs formulations was evaluated by MTT and trypan blue assay on PC12 cells. PC12 cell line presents different physiological properties of dopaminergic neurons and represents a good model to study the neurodegenerative events (Karten et al., 2005). MTT and Trypan blue assay have allowed to identify the optimal formulation and concentrations that do not induce cytotoxicity. By employing nonionic edge activators, non-toxic nanovescicles are obtained; on the contrary GEN-TF3 containing the anionic Na-DOC is highly cytotoxic on PC12 cells. Generally, nonionic surfactants are preferred with respect anionic, amphoteric or

cationic counterparts as they impart to vesicles superior benefits with respect to stability, compatibility and toxicity (Kumar and Rajeshwarrao, 2011).

The encapsulation of GEN in the transferosomes (GEN-TFs) improves its performance compared to GEN. Among GEN-TFs tested, it is highlighted a protective effect of the GEN-TF2. The several formulations have different edge activators and, these differences in their composition could explain the different behavior of the drug delivery systems in PC12 cells. GEN-TF2 and GEN-TF4 consisting of lipophilic surfactants, present different effect in addition with H_2O_2 (Maioli et al., 2015). GEN-TF2 shows a evident protection from the oxidative insult. GEN-TF1 has hydrophilic characteristics, and preserves the cell viability in PC12 cells damaged. On the basis of these considerations, it was investigated about the GEN-TF2 formulation that showed the greatest protective effect.

In the uptake studies, GEN-TF2 shows to be a good drug delivery system to improve the Genistein internalization by cells if compared with GEN (Li et al., 2016). The GEN-TF2 is able to significantly attenuate the reactive oxygen species overproduction induced by oxidative stress compared to GEN and, it could be considered a ROS scavenger comparable to endogenous antioxidant N-acetyl-L-cysteine (NAC) (Tavakkoli et al., 2014). GEN-TF2 is able to restore the cell cycle phases modified by hydrogen peroxide alone or with GEN (Liu H et al., 2017). Moreover, the studied formulation shows a significant anti-apoptotic effect: in fact GEN-TF2 reduces the apoptotic events on PC12 cells exposed to hydrogen peroxide. The antioxidant and anti-apoptotic effects of the considered formulation could be due to its best performance of uptake from the cell line.

GEN-TF2 thus could be an efficient drug delivery system to improve the CNS delivery of GEN by the nose-to-brain route by enhancing drug passage via olfactory nerve pathway (Pardeshi and Belgamwar, 2013).

Conclusions

From the results shown, it can be stated that suitable delivery systems for the phytoestrogen genistein are obtained. The composition of transfersomes is responsible of different technological properties such as deformability, size and *in vitro* permeation. The transfersomes obtained, except GEN-TF3, show less cytotoxicity compared to GEN on PC12 cells.

The potential risk of apoptotic death induced by the oxidative injury, and the ROS level increase could lead to neurotoxic effects. The GEN-TF2 formulation, studied in this paper, is able to reduce the amount of apoptotic cells generated by H_2O_2 treatment. These obtained data confer to GEN-TF2 antioxidant and antiapoptotic activities. Therefore, it could be considered a suitable intranasal delivery system as therapeutic adjuvant in oxidative stress-related neurodegenerative diseases: this hypothesis could be supported by performing in vivo studies on NDs animal model.

Conflict of interest

The authors report no conflicts of interest in this work.

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Table 1

Composition of TFs and GEN-TFs, weight of solid components. Ultra-pure water has been added up to 50 ml in order to have the final GEN concentration of 0.4 mg/ml (GEN-TF1 and GEN-TF2) or 0.2 mg/ml (GEN-TF3 and GEN-TF4).

Formulation	Kind of edge activator	Phospholipid (mg)	Edge activator (mg)	GEN (mg)
TF1	Tween 80	686	294	-
GEN-TF1	Tween 80	686	294	20
TF2	Span 80	686	294	-
GEN-TF2	Span 80	686	294	20
TF3	Na-DOC[a]	693	297	-
GEN-TF3	Na-DOC[a]	693	297	10
TF4	Span 20	693	297	-
GEN-TF4	Span 20	693	297	10

Table 2

Characterization of TFs and GEN-TFs

Formulations	Vesicle size (nm ± SD)[1]	PDI (± SD)[1]	LE (%±SD)	Relative deformability (sec ± SD)
TF1	74±0.6	0.337±0.006		33±0.01
GEN-TF1	70.5±0.75	0.183±0.015	98.73±0.31*	240±0.2#
TF2	176.6±1.5[a,b,c]	0.442±0.001	-	0
GEN-TF2	132.8±1.35[c]	0.310±0.029	40.36±0.23	0
TF3	37.8±0.5[d]	1.286±0.065	-	8.99±0.4
GEN-TF3	30.3±3.5[d]	1.320±1.200	95.06±0.09*	9.0±0.22
TF4	117.9±1.6[a,b]	0.372±0.019	-	0
GEN-TF4	119.3±0.75	0.321±0.010	95.50±0.005*	0

[1]Mean diameter of TFs and GEN-TFs: [a]P<0.05 *vs* TF1; [b]P<0.05 *vs* TF3; P<0.05: [c]TF2 *vs* GEN-TF2; [d]TF3 *vs* GEN-TF3. LE %: *P<0.05 *vs* TF2. Relative deformability (sec): #P<0.05 *vs* TF1

Figure legends

Fig. 1 *In vitro* permeation profiles of GEN-TFs through octanol-saturated membranes. *P<0.05 GEN-TF1 *vs* GEN-TF3 and GEN-TF2. # P<0.05 GEN-TF3 *vs* GEN-TF2.

Fig. 2 Screening of formulations of GEN-TFs on PC12 cells: MTT analysis was performed on PC12 cells 24h after exposure to GEN-TF1, 2, 3, 4 (30 µM and 50 µM) in comparison with Control (*P<0.05 vs Control).

Fig. 3 Effect of different concentrations of GEN-TF1, -TF2, -TF4 on viability of PC12 cells exposed to hydrogen peroxide. MTT assay **(a)** and Trypan Blue analysis **(b)** were performed on PC12 cells 24h after exposure to GEN-TF1, -TF2, -TF4 30 µM alone or in presence of H_2O_2 75 µM (*P < 0.05 vs Control) (#P < 0.05 vs H_2O_2 75 µM) (§P < 0.05 vs GEN 30 µM + H_2O_2 75 µM) (°P < 0.05 vs .GEN-TF2 30 µM + H_2O_2 75 µM)

Fig. 4 Effect of GEN-TF2 30 µM on LDH release in H_2O_2 -treated PC12 cells after 24h exposure. The results are expressed as % LDH release (*P < 0.05 vs Control) (#P < 0.05 vs H_2O_2 75 µM) (§P < 0.05 vs GEN 30 µM + H_2O_2 75 µM).

Fig. 5 Cell cycle analysis by flow cytometry after treatment with GEN-TF2 30 µM for 24h in PC12 cells exposed to H_2O_2. Histograms representation of the distribution of the cell population during the cell cycle phases.

Fig. 6 ROS content in hydrogen peroxide exposed-PC12 cells treated with GEN-TF2 30 µM for 24h. Fluorescent cells (%) as a function of ROS produced. (*P < 0.05 vs Control) (#P < 0.05 vs H_2O_2 75 µM) (§P < 0.05 vs GEN 30 µM + H_2O_2 75 µM).

Fig. 7 Cell cycle analysis by flow cytometry after treatment with GEN-TF2 30 µM for 24h in PC12 cells exposed to H_2O_2. Schematic representation of the distribution of the cell population during the cell cycle phase.

Fig. 8 Apoptosis analysis by flow cytometry performed on PC12 cell culture 24h exposed to hydrogen peroxide in presence of GEN-TF2 30 µM (*P < 0.05 vs Control) (#P < 0.05 vs H_2O_2 75 µM) (§P < 0.05 vs GEN 30 µM + H_2O_2 75 µM).

Fig. 9 Fluorescence Microscopy (Hoechst 33342) of PC12 cells nuclei exposed to GEN-TF2 and H_2O_2 for 24h. **A:** Control; **B:** H_2O_2 75 µM; **C:** GEN-TF2 30 µM + H_2O_2 75 µM.

Figure 1

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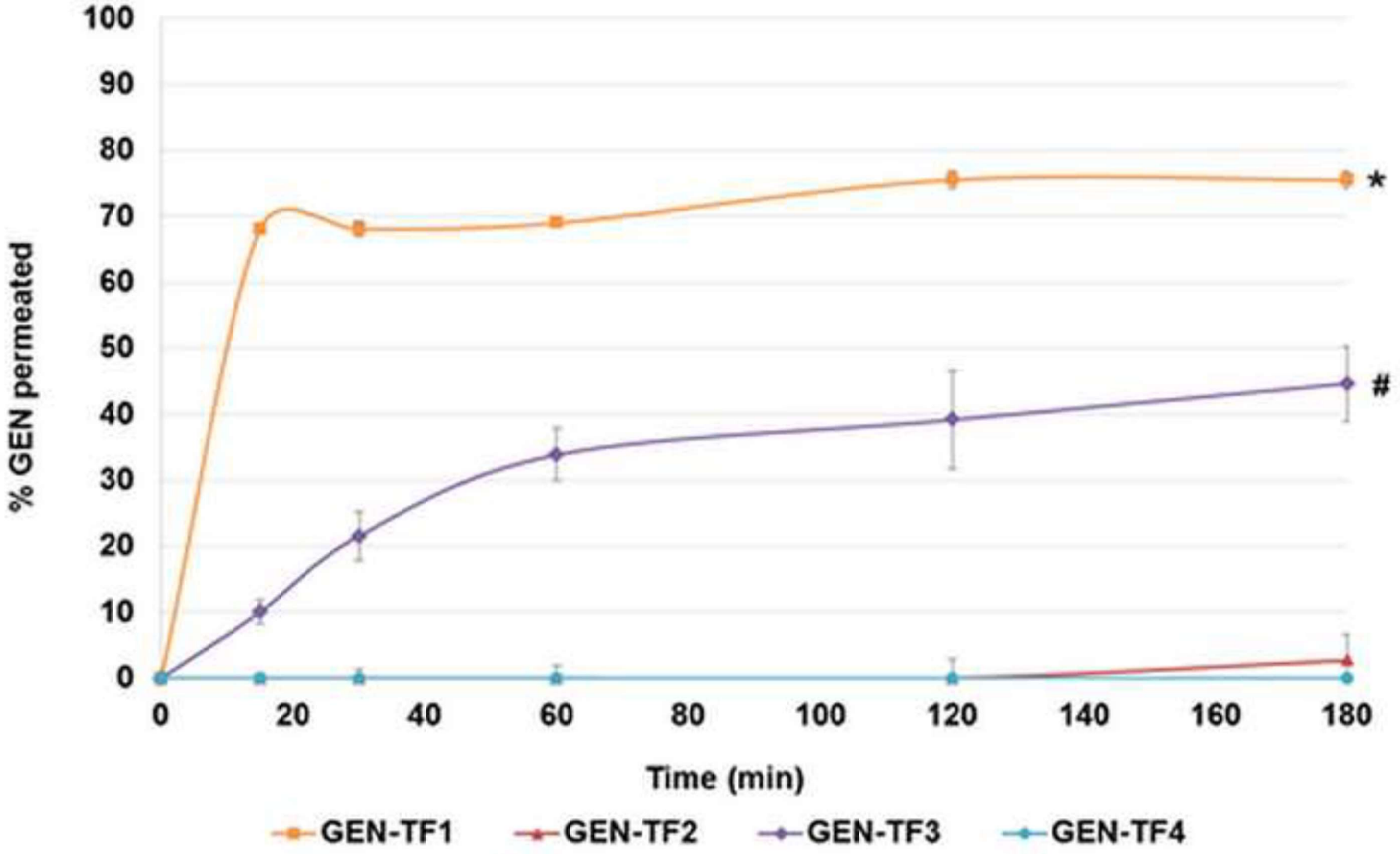

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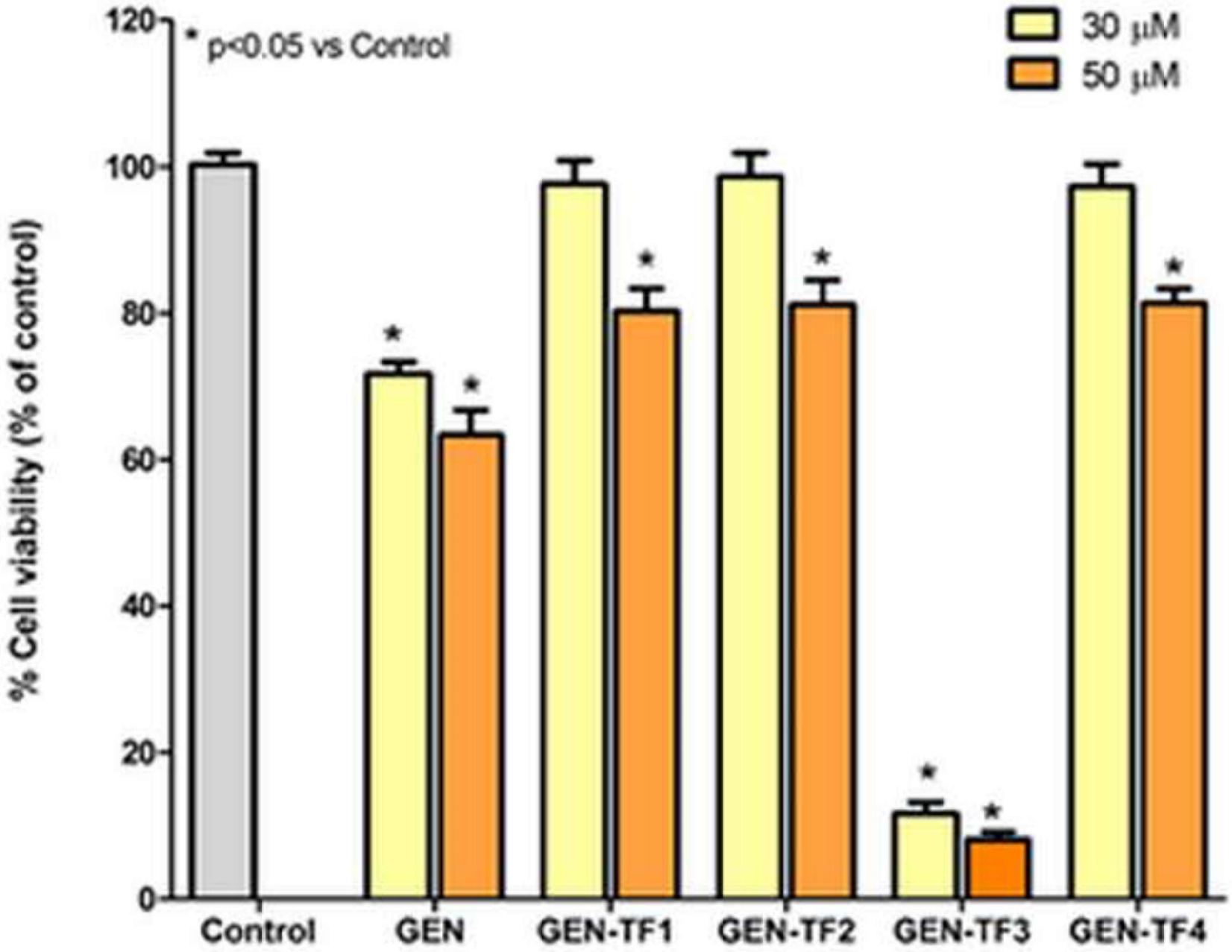

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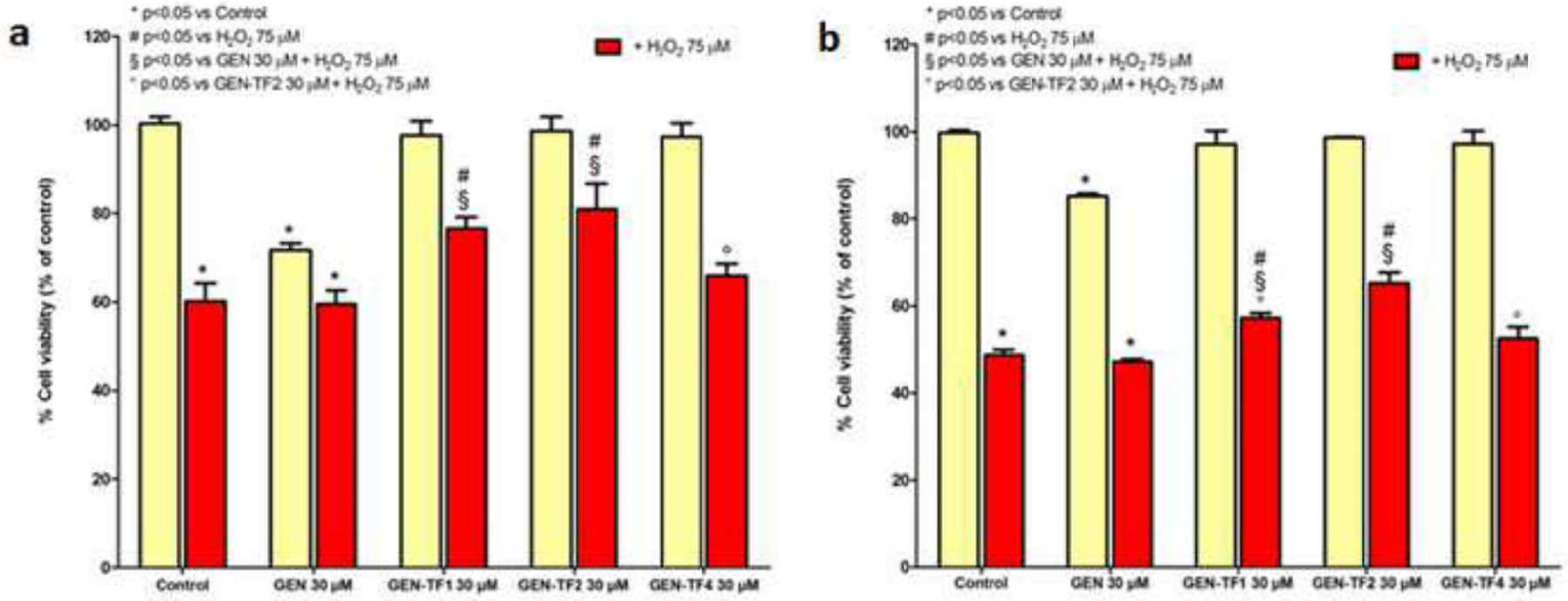

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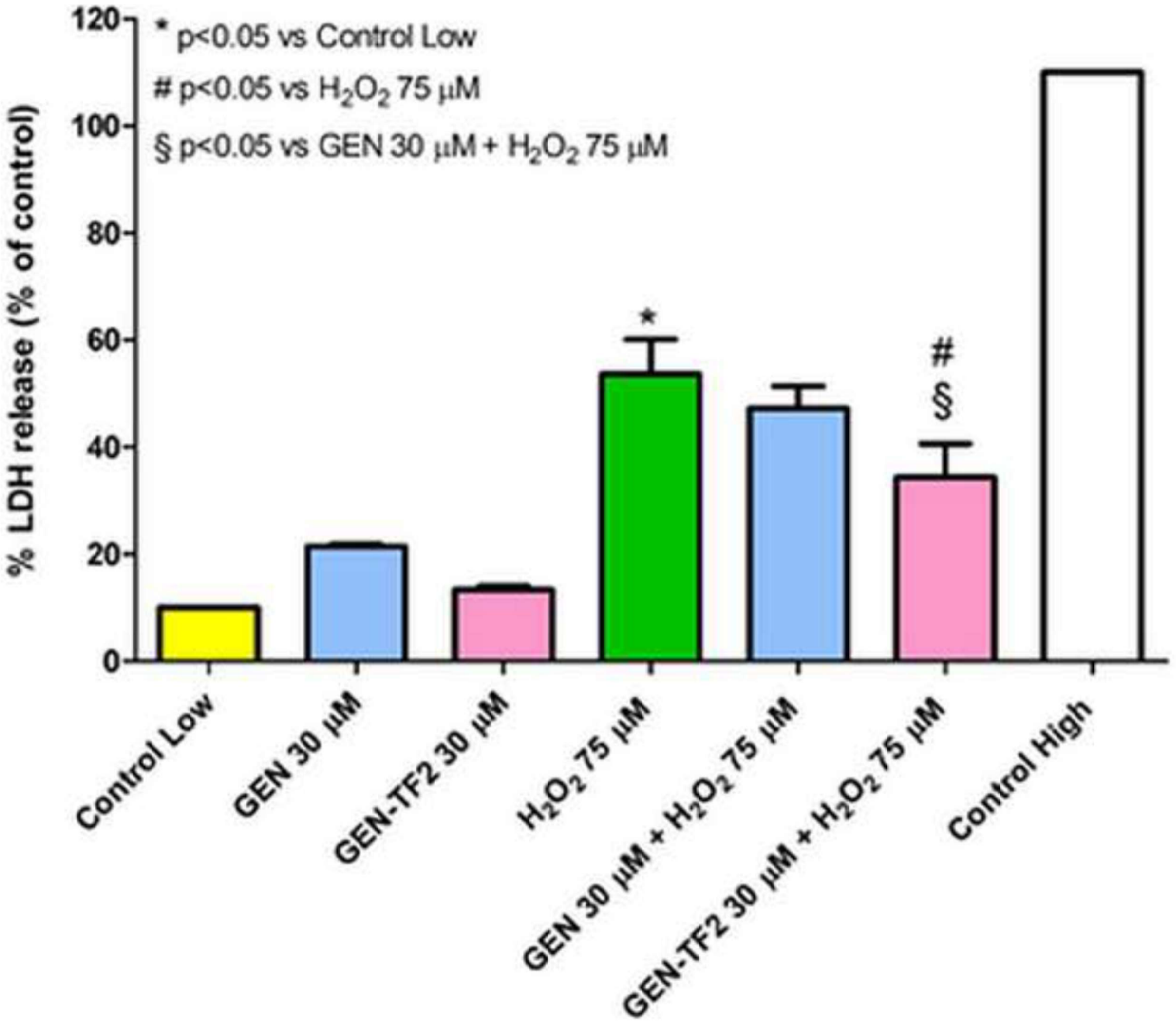

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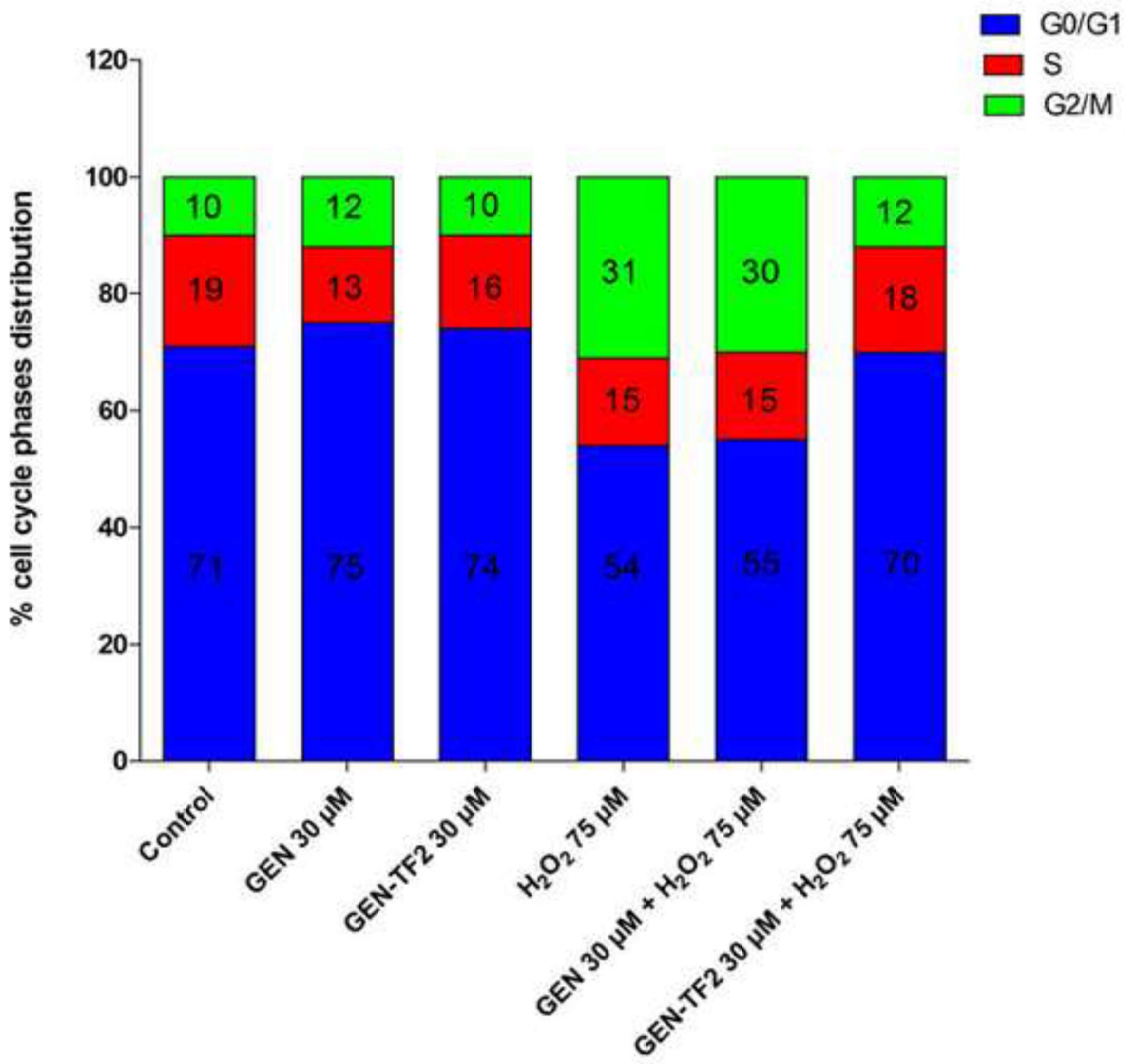

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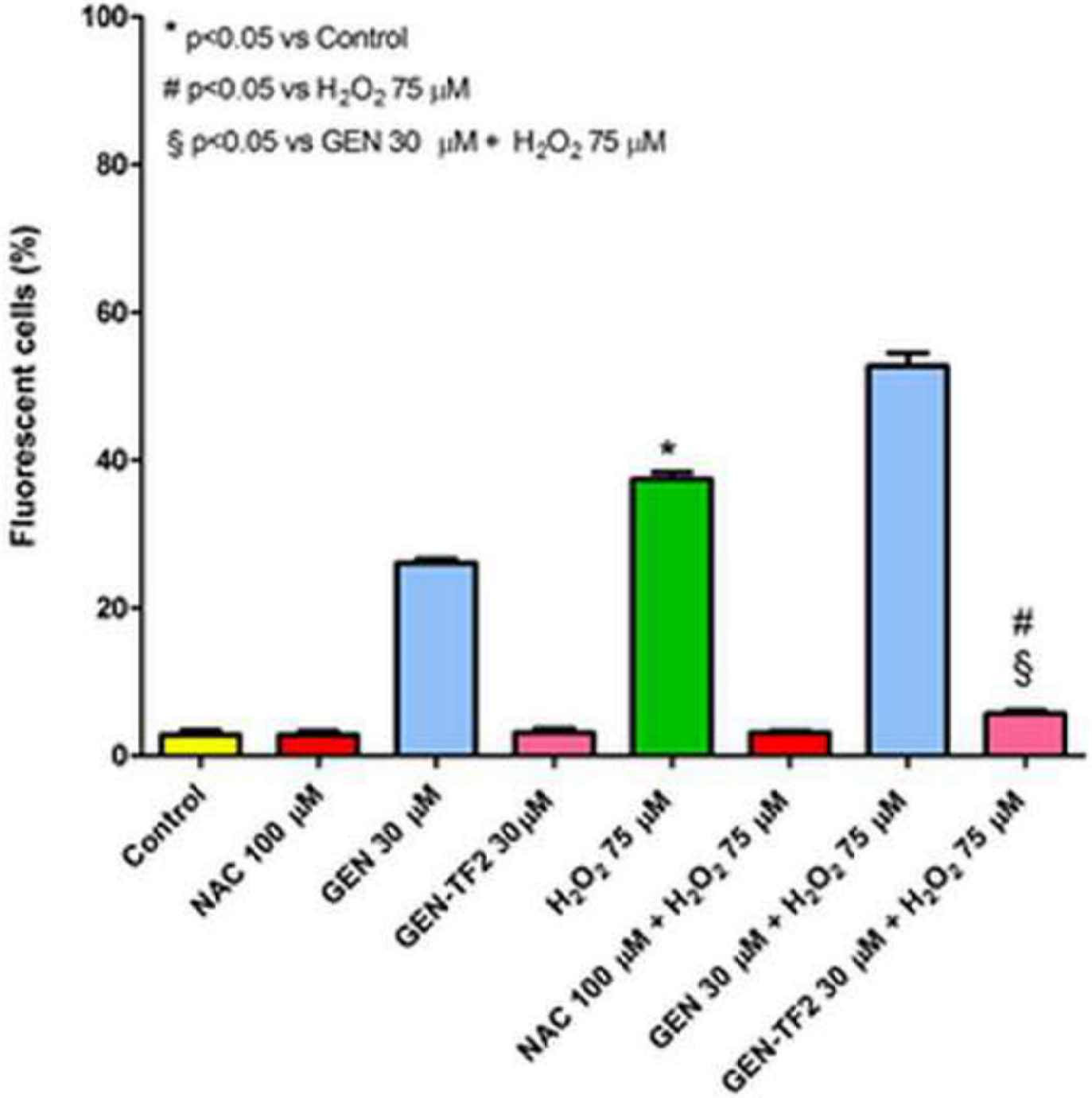

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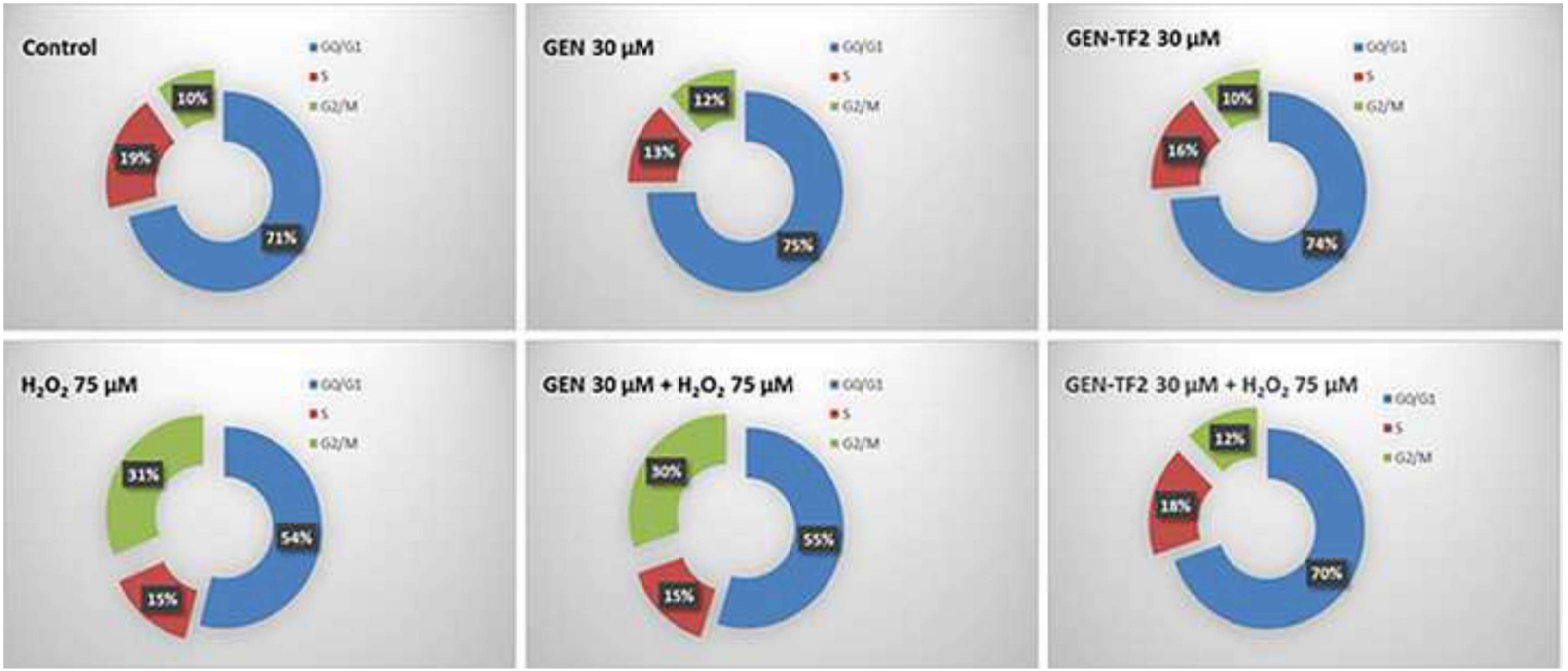

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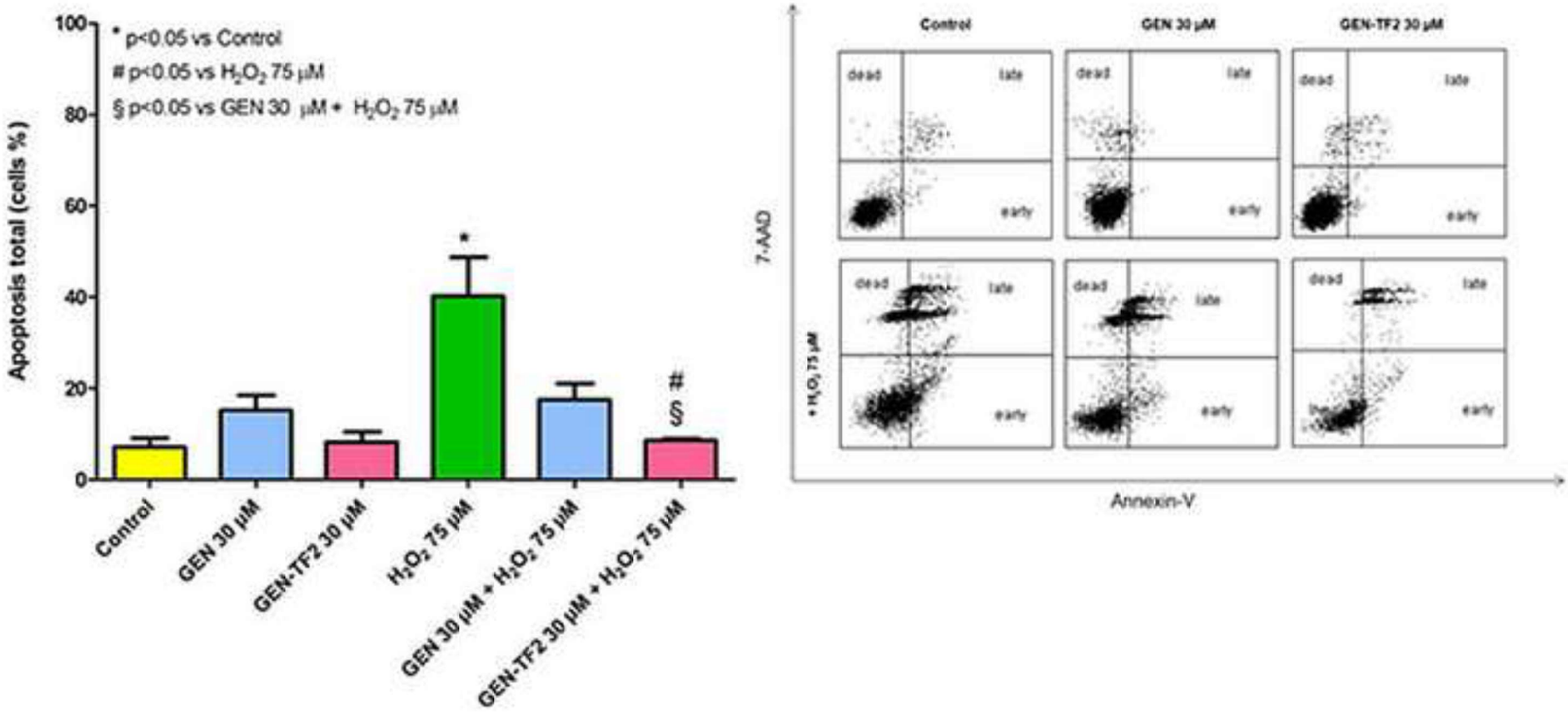

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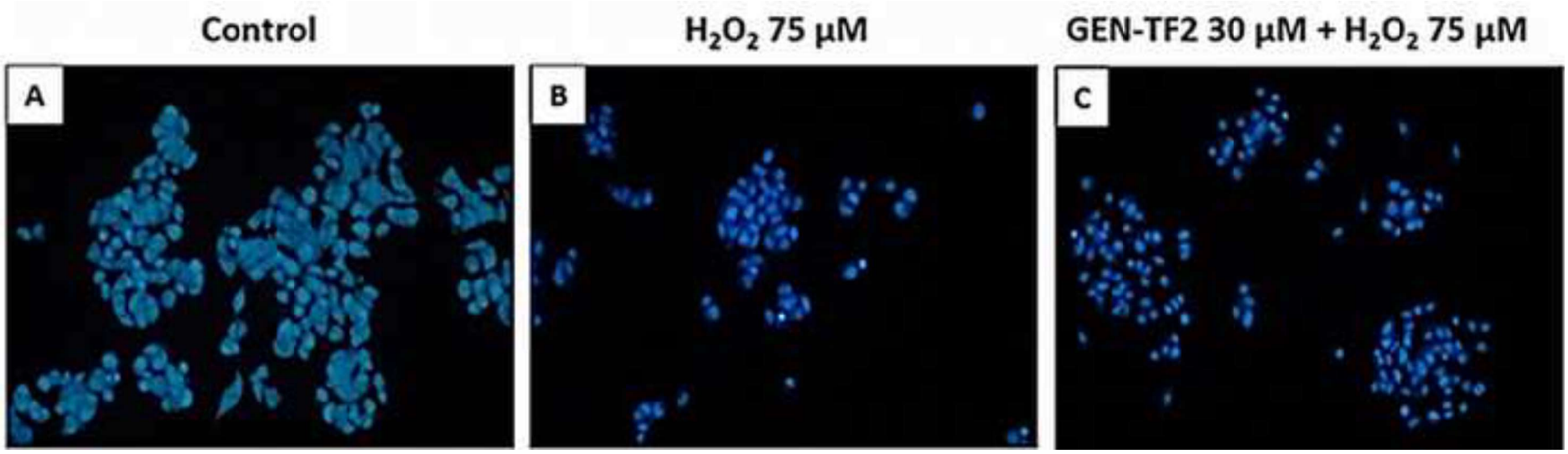

Appendix. Supplementary materials

Genistein-loaded transferosomes as adjuvant in the oxidative stress-related neurodegenerative diseases

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S1. Materials and Methods

S1.1 Transmission electron microscopic observation

The morphology of transfersomes was evaluated by Transmission Electron Microscopy (TEM), using a Philips CM10 (Eindhoven, NL) instrument. Before observation, transferosome suspensions were diluted with deionized filtered water (1:10 dilution) and were negatively stained with osmium tetroxide solution (1% v/v). A drop of stained transfersomes aqueous suspensions was placed on Formvar-coated copper grid and air-dried before observation.

S.1.2 Evaluation of the GEN loading

The quantitative determination of GEN in the GEN-TFs was performed using a modified, rapid and sensitive high performance liquid chromatographic method (de Zampieri et al., 2013).The binary mobile phase consisted of acetonitrile and a 25mM (350µl/250ml) acetic acid solution ratio 50:50 (v/v) which was filtered through 0.45µm nylon membrane filters (Sartorius, Goettingen, Germany) prior to use. The mobile phase was pumped isocratically at a flow rate of 1.2 ml/min at ambient temperature. Peak heights were recorded at 260 nm.
Formulations (50 µl) were added to an appropriate volume of methanol (950 µl), able to completely dissolve the lipid vesicles, thus extracting the drug. The solution was mixed with vortex for 1 min, then analyzed by HPLC. The GEN content was determined by data extrapolation using a previously prepared calibration curve ($y= 600380x+111006$; $R^2= 0.9996$), obtained using GEN standard solutions with a concentration range of 0.5-20 mg/l. Drug loading efficiency (LE) percentage (n=3±SD) was calculated (Langasco et al., 2017).

S1.3 Evaluation of the stability

The different TFs and GEN-TFs were stored at 25°C and 4°C, in order to evaluate their physical stability as function of the time and storing conditions. Formulations were observed macroscopically, regarding any change in the overall homogeneity of the system; moreover, dimensional analyses were performed after1, 2 and 3 months from the preparation. Size and PDI of formulations at different storage temperature and along the considered time were compared and expressed as mean ±SD (n=3).

S1.4 Relative deformability

The elasticity of the bilayer of the various TFs and GEN-TFs was tested by a modified extrusion measurement (El Zaafarany et al., 2010). A known volume of formulations (10 ml) was extruded through inorganic membranes (Whatman® Anodisc, 0.22 μm pore size) under a vacuum system. The elasticity was determined by measuring the time taken for the total extrusion of formulations through the membrane. The experiments were carried out in triplicates to obtain an average value.

S.2 Results

S2.1 Transmission electron microscopic observation

TFs appear as spherical and multilamellar vesicles, with the lamellae of vesicles evenly spaced to the core, and with uniform size distribution (Fig. S1, a); no aggregation irregularities are observed. On the contrary, GEN-TFs are unilamellar vesicles with clear internal core covered by a phospholipid layer (Fig. S1, b).

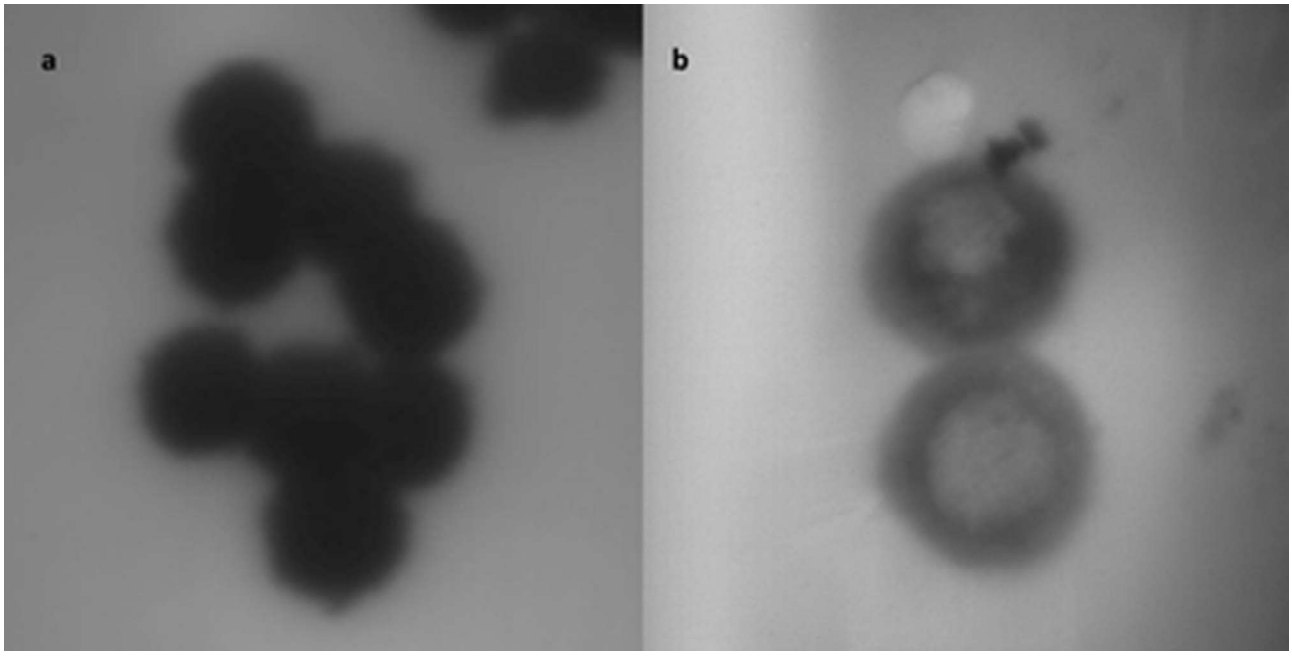

Fig. 1. TEM image of TF2 (a) and GEN-TF2 (b) (Magnification 39000×).

S.2.2 Evaluation of the GEN loading

The GEN content into the formulations was assessed in order to confirm the efficacy of the preparation method and the effective loading of the drug into the vesicles, excluding any physical alteration phenomena. It is known, indeed, that GEN is sensitive to light, heat and oxidation (Aditya et al., 2013). Results are shown in Table 2. The GEN content measured is very similar to the theoretical one (almost 100%) for all formulations, except for GEN-TF2 ($P<0.05$), as it might be expected. As previously mentioned, GEN-TF2 leaves a residual portion of the film after the hydration step, suggesting that an amount of GEN could be entrapped into the film layer and it could not be completely incorporated into the vesicular dispersion. To this respect, the quantitative analysis carried out on the residual thin film confirms percentages of GEN equivalent to 50%, corresponding to the missing amount in the formulation. It can be hypothesized that this behavior is ascribed to the edge activator nature, since the lowest HLB value (4.3) might cause the formation of a less flexible film, difficult to hydrate.

S2.3 Evaluation of the stability

Fig. S2 shows the stability trends of all TFs and GEN-TFs, in terms of vesicle size within the time, as function of the storage conditions. TF1 and GEN-TF1 generally exhibits a good stability from the preparation to the first month (Fig. S2a). During the third month, it can be appreciated a significant increase of the mean diameter, at room temperature and at 4°C ($P<0.05$). TF2 demonstrates a general good stability; however,GEN-TF2 undergoes an increase of the dimensions (Fig. S2b), going from about 130 nm at the preparation time to about 200 nm after 3 months ($P<0.05$). This trend is more evident for the room temperature storage, because no statistical differences occur between 1 to 3 months for GEN-TF2 samples stored at 4°C ($P<0.05$). PDI values between 0.2 and 0.3 of these two formulations (data not shown) indicate a great homogeneity, which is not significantly altered during the time ($P>0.05$). The dimensional values of TF3 and GEN-TF3 (Fig. S2c) suggest a significant vesicular aggregation within the time ($P<0.05$), regardless the storing conditions. Moreover, the high PDI values confirmed an inhomogeneous formulation in terms of mean diameter either after the preparation or after few months. The analyses on TF4 (Fig. S2d) reveal a relatively stable formulation, especially at 4°C, although an important increase of the mean size can be observed for the GEN-loaded formulation (GEN-TF4) during the time ($P<0.05$), even when stored at 4°C.

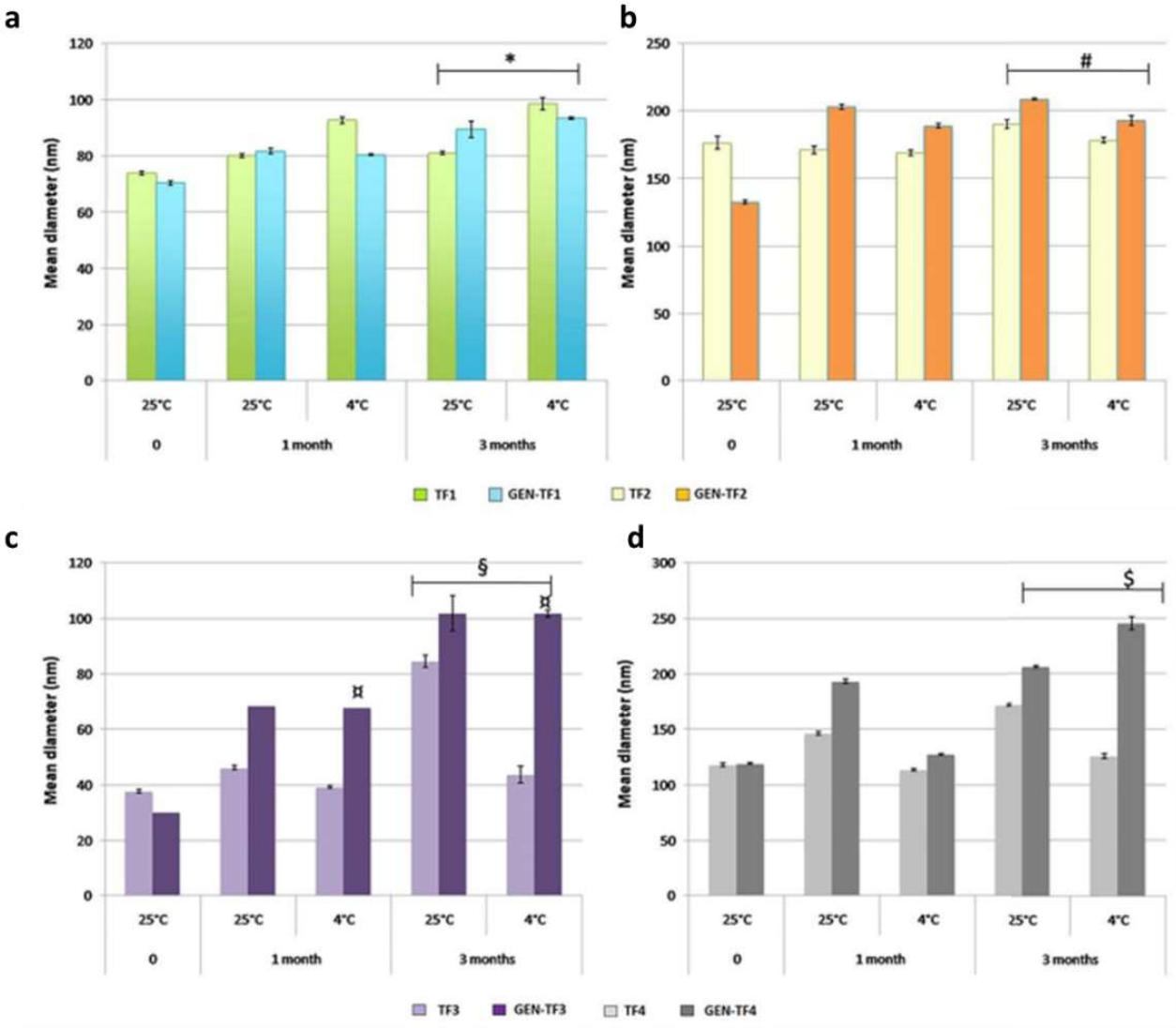

Fig. 2. Change in the particle size of TF1 and GEN-TF1 (a), TF2 and GEN-TF2 (b), TF3 and GEN-TF3 (c), TF4 and GEN-TF4 (d) after storage at 25°C and 4°C for 1 and 3 months. *P<0.05 *vs* TF1 and GEN-TF1 at 0 time and 25°C; #P<0.05 *vs* TF2 and GEN-TF2 at 0 time and 25 °C; §P<0.05 *vs* TF3 and GEN-TF3 at 0 time and 25°C; $ P<0.05 *vs* TF4 and GEN-TF4 at 0 time and 25°C; ¤ P<0.05: GEN-1 TF3 at 4°C and 3 months *vs* GEN-TF3 at 4°C and 1 month.

S1.4 Relative deformability

Table 2 also lists the relative deformability measured, for both TFs and GEN-TFs. TF2 and TF4 are not able to be extruded through the membrane while TF1 and TF3 show an elasticity of the bilayer than the lipophilic counterparts. The GEN loading significantly affects the relative deformability of TF1 (P<0.05). Results obtained from this study can be linked to the edge activator type, the vesicle size and the presence of GEN. Specifically, TF1, GEN-TF1, TF3 and GEN-TF3 possess a high bilayer elasticity, probably due to the presence of hydrophilic edge activators that leads to reduced dimensions, thus an easier crossing of the membrane. On the other hand, TF2 and TF4 (with lipophilic edge activators) possess no deformability, regardless the GEN loading, assuming that the presence of lipophilic molecules (edge activators and drug)

negatively influence the deformability of formulations by rendering vesicles more rigid (El Zaafarany et al., 2010).

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