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## Molecular and Cellular Pharmacology

## Oxidative DNA damage protection and repair by polyphenolic compounds in PC12 cells

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## ABSTRACT

Biological systems are frequently exposed to excessive reactive oxygen species, causing a disturbance in the cells natural antioxidant defence systems and resulting in damage to all biomolecules, including nucleic acids. In fact, oxidative DNA damage is described as the type of damage most likely to occur in neuronal cells. In this study, three polyphenolic compounds, luteolin, quercetin and rosmarinic acid, were investigated for their protective effects against oxidative DNA damage induced in PC12 cells, a neuronal cell model. Although luteolin and quercetin prevented the formation of strand breaks to a greater extent than rosmarinic acid, this last one presented the highest capacity to repair strand breaks formation. In addition, rosmarinic acid was the only compound tested that increased the repair of oxidized nucleotidic bases induced with the photosensitizer compound [R]-1-[(10-chloro-4-oxo-3-phenyl-4H-benzo[a]quinolizin-1-yl) carbonyl]-2-pyrrolidine-methanol (Ro 19-8022). The activity of repair enzymes was indicated by the *in vitro* base excision repair assay, using a cell-free extract obtained from cells previously treated with the compounds to incise DNA. The protective effect of rosmarinic acid was further confirmed by the increased expression of OGG1 repair gene, observed through *real time* RT-PCR. The data obtained is indicative that rosmarinic acid seems to act on the intracellular mechanisms responsible for DNA repair, rather than by a direct effect on reactive oxygen species scavenging, as deduced from the effects observed for luteolin and quercetin. Therefore, these results suggest the importance of these polyphenols, and in particular rosmarinic acid, as protectors of oxidative stress-induced DNA damage that commonly occurs in several pathological conditions, such as neurodegenerative diseases.

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## 1. Introduction

In living cells, when the formation of intracellular reactive oxygen species exceeds the cells' antioxidant capacity, oxidative stress can arise, resulting in damage to cellular macromolecules such as proteins, lipids and DNA (Nordberg and Arner, 2001; Valko et al., 2007). DNA is a particularly sensitive cellular target because of the potential to create cumulative mutations that can disrupt cellular homeostasis. In this case, the reactive oxygen species can lead to the formation of single and double-strand breaks, as well as induce chemical and structural modifications to purine and pyrimidine bases, and also to 2'-deoxyribose (Powell et al., 2005; Hazra et al., 2007).

Oxidative DNA damage has been considered as an important promoter of cancer, besides being implicated in the normal process of aging (Bjelland and Seeberg, 2003). In addition, according to some authors, it is regarded as the type of damage most likely to occur in neuronal cells (Fishel et al., 2007). However, this kind of DNA damage is predominantly corrected by the base excision repair (BER) pathway

(Coppede et al., 2007), although certain types of oxidative lesions also appear to be repaired by nucleotide excision repair (NER) (Dusinska et al., 2006) and mismatch repair (MMR) (Neri et al., 2005). BER is a generic mode of repair, whose first steps are the recognition of damaged bases by specific DNA glycosylases, hydrolysis of the glycosidic bond between base and deoxyribose and incision of the affected DNA strand by an apurinic/apyrimidinic (AP) endonuclease at the resulting abasic site, thus creating a DNA single-strand break (Burkle, 2006; Collins and Gaivão, 2007). The most important enzymes involved in DNA repair are 8-oxoguanine DNA glycosylase 1 (OGG1) and AP endonuclease 1 (APE1). In mammals, OGG1 is responsible for the removal of 8-oxoguanine, a lesion that arises through the incorporation, during DNA replication, of 8-oxo-dGTP formed from oxidation of dGTP by reactive oxygen species. This enzyme also has an AP lyase activity, which is slow and limits the overall rate of repair (Alamo et al., 1998; Dodson and Lloyd, 2002). APE1, by its turn, is an AP endonuclease that bypasses the AP lyase activity of OGG1, enhancing OGG1 turnover, thus having an important role in the regulation of base excision repair of oxidative DNA damage (Hill et al., 2001; Vidal et al., 2001).

Antioxidant activity, as well as interaction with several enzymes and synergy with other antioxidants, has been recognized to polyphenolic compounds (Horvathova et al., 2005), which are secondary

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plant metabolites with numerous other biological activities (Rice-Evans et al., 1996; Hollman and Katan, 1999; Skibola and Smith, 2000). In the present study, attention was given to the flavonoids quercetin and luteolin, and to the phenolic acid, rosmarinic acid (Fig. 1). Quercetin is one of the most abundant natural flavonoids and can be found in onion, tea and apple, for example (Scalbert et al., 2005). Luteolin, which differs from quercetin by having one less hydroxyl group in the C-ring of its molecular structure (Fig. 1), is found in high celery, green pepper and chamomile (Gutierrez-Venegas et al., 2006). Both quercetin and luteolin, which by their chemical nature are antioxidants, have been associated to the prevention of cancer, diabetes, osteoporosis, as well as cardiovascular and neurodegenerative diseases, among others (Hollman and Katan, 1999; Aherne and O'Brien, 2000; Scalbert et al., 2005). Rosmarinic acid is present in many plants, such as rosemary (Petersen and Simmonds, 2003) and its therapeutical value has been attributed to its antioxidant, anti-inflammatory, anti-bacterial and anti-viral properties (Petersen and Simmonds, 2003; Chlopcíková et al., 2004; Kim et al., 2005; Iuvone et al., 2006). Neuroprotective effects have also been described for all of these compounds (Gelinas and Martinoli, 2002; Sasaki et al., 2003; Iuvone et al., 2006), suggesting their potential protective role in neurodegenerative diseases. Moreover, studies using some of these compounds have demonstrated their ability to protect different cell types against oxidative DNA damage (Noroozi et al., 1998; Duthie and Dobson, 1999; Horvathova et al., 2005; Lima et al., 2006). However, whether they act by enhancing DNA repair or simply by preventing oxidative DNA damage is still unknown.

In this work, we evaluated the potential of these polyphenolic compounds to protect PC12 cells against oxidative DNA damage, by using the Comet assay. In addition, by addressing specific repair enzymes, we aimed to further characterize that protection and explore the involvement of those compounds in DNA repair mechanisms. The PC12 cell model was used as a simple model in which a correlation between oxidative stress and neurodegeneration, characteristic of Parkinson's or Alzheimer diseases, has been established by several authors (Piga et al., 2005; Guan et al., 2006; Jung et al., 2007).

## 2. Materials and methods

### 2.1. Chemicals

Quercetin and rosmarinic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Luteolin was purchased from Extrasynthese

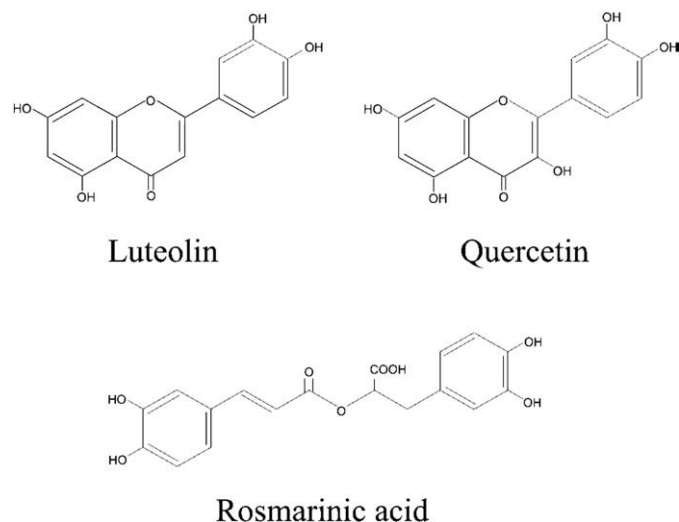


Fig. 1. Chemical structures of the polyphenolic compounds used in this study: luteolin, quercetin and rosmarinic acid.

(Genay, France). The polyphenolic compounds were dissolved in DMSO, aliquoted and maintained frozen at  $-20^{\circ}\text{C}$  until usage. Each aliquot was thawed only once.

RPMI-1640 cell culture medium, dimethyl sulfoxide (DMSO), EDTA, trypsin, *tert*-butyl hydroperoxide were also purchased from Sigma-Aldrich (St. Louis, MO, USA).

Foetal bovine serum (FBS) was from BioChrom KG (Berlin, Germany); horse serum donor herd was purchased from Gibco (Paisley, UK). [R]-1-[(10-chloro-4-oxo-3-phenyl-4*H*-benzo[*a*]quinolizin-1-yl) carbonyl]-2-pyrrolidine-methanol (Ro 19-8022), used to induce specific DNA damage, was kindly provided by Hoffman La-Roche (Basel, Switzerland). Primers specific for 18S, OGG1 and APE1 genes were synthesized by STAB-VIDA (Oeiras, Portugal). Qiagen RNeasy total RNA isolation kit was purchased to Qiagen (Hilden, Germany). Superscript Reverse Transcriptase III kit was obtained from Invitrogen (USA). Power SYBR Green master mix was acquired from Applied Biosciences (Cheshire, UK).

### 2.2. PC12 cell culture and treatment conditions

PC12, a neuronal cell line established from a rat adrenal pheochromocytoma (Greene and Tischler, 1976) was used in this study. When grown in serum-containing medium, these cells divide and resemble precursors of adrenal chromaffin cells and sympathetic neurons, being able to release dopamine (Sasaki et al., 2003). In addition, they are a user-friendly cell model with some advantages over primary cultured neuronal cells, including the homogeneity of the cell population (Colognato et al., 2006; Silva et al., 2008). Cells were cultured in suspension in 75  $\text{cm}^2$  flasks, in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated horse serum, 5% (v/v) heat-inactivated foetal bovine serum and 1% (v/v) of an antibiotic/antimycotic solution. Cultures were maintained in a humidified incubator containing 95% air and 5%  $\text{CO}_2$ , and passed twice a week. Before each assay, the cell aggregates were carefully disrupted by gently pipetting and the separated cells plated in poly-*D*-lysine-coated multiwells, at a density of  $2.5 \times 10^5$  cells/ $\text{cm}^2$ , for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and at a density of  $5 \times 10^5$  cells/well for the other studies. After plating, cells were left for adhesion overnight. The polyphenols were either pre-incubated for the indicated periods of time or added simultaneously with the deleterious stimuli.

L929 cells, used as substrates in the *in vitro* base excision repair assay, were routinely grown in 75  $\text{cm}^2$  tissue culture flasks in DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) of an antibiotic/antimycotic solution containing 10,000 U of penicillin, 10 mg streptomycin and 25  $\mu\text{g}$  amphotericin B per ml.

### 2.3. Analysis of cell survival

Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction test, as previously described (Silva et al., 2006). A volume of 0.5 ml MTT (final concentration 0.5 mg/ml, in Krebs medium, pH=7.4), prepared just before usage and maintained in the dark, was added to the PC12 cells, at a density of  $2.5 \times 10^5$  cells/ $\text{cm}^2$ . Absorbance was read at 570 nm in a multiplate reader (Spectramax 340PC). The survival of PC12 cells was expressed as the percentage of OD towards control cells, containing the same amount of the compounds' solvent, DMSO.

### 2.4. Single cell gel electrophoresis (Comet assay)

The protection against oxidative DNA damage conferred by the polyphenols herein studied was assessed using the Comet assay, in which the strand breaks present in the DNA of nucleoids, obtained after lysis of gel-embedded cells, migrate towards the anode during an electrophoresis in alkali conditions, yielding an image that, after

staining with a fluorescent dye, looks like a comet, hence its name. This method was performed as previously described (Tice et al., 2000), with the following modifications. Cells were plated at a density of  $7.5 \times 10^5$  cells per well and left to adhere overnight. Oxidative DNA damage was induced in the presence of either 200  $\mu\text{M}$  *t*-BHP (for strand breaks assessment) or 0.6  $\mu\text{M}$  Ro 19-8022, in order to measure oxidized purines. Cells were then trypsinized, resuspended in phosphate buffered saline (PBS) and counted. About 50,000 cells were centrifuged at  $\sim 1500 \times g$ , for 1 min, in an Eppendorf 5415C centrifuge. Supernatants were discarded and pellets mixed with 100  $\mu\text{l}$  of low melting point agarose 0.5% (w/v) in PBS, at 37 °C, and spread on slides previously coated with normal melting point agarose for 10 min, at 4 °C. The slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, with 1% Triton X-100 v/v added at the time of buffer preparation) at 4 °C for a minimum of 2 h. Slides were then rinsed with distilled water and immersed in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13), in an horizontal electrophoresis tank, at 4 °C for 40 min, to allow alkaline unwinding. Electrophoresis was carried out at 4 °C, under alkaline conditions for 20 min at 1 V/cm. Finally, slides were neutralized by washing three times (5 min each) with 0.4 M Tris, pH 7.5, and fixed with absolute ethanol (two washes).

Comets were stained with ethidium bromide (10  $\mu\text{g}/\text{ml}$  in PBS) and analyzed under a fluorescence microscope. Comet quantification was performed through either of two ways: visual scoring, a method in which comets are classified into one of five classes of damage (from 0 to 4) in 100 nucleoids (range of score: 0–400); and/or through a computer-assisted image analysis (TriTek CometScore™ Freeware v1.5), by measuring the percentage of DNA in the tail. The method identifies DNA strand breaks.

Cells repair capacity (RC) was determined by using the following formula:

$$\text{RC} = 100 \times [(D_0X - D_rX) / D_0X],$$

where  $D_0X$  represent DNA damage before the recovery period in the condition X and  $D_rX$  represent DNA damage after a recovery period, for the same condition. The increase in cells' repair capacity induced by the polyphenolic compounds was obtained by subtracting the percentage of repair observed in the presence of the deleterious stimulus alone, to the percentage of repair in the presence of the compounds and the deleterious stimulus.

### 2.5. Measurement of oxidized purines

Occurrence of oxidized bases were measured by using the bacterial DNA repair enzyme formamidopyrimidine DNA glycosylase (FPG), which recognizes oxidized purines, creating breaks at those sites (Duthie and Dobson, 1999).

Cells were treated with the photosensitizer compound Ro 19-8022 (a kind gift from Hoffmann La-Roche, Basel, Switzerland). A stock of a 1 mM concentration was prepared in 70% ethanol and stored in small aliquots at  $-20$  °C. The working solutions were prepared immediately before use by diluting this stock solution with PBS (132 mM NaCl, 4 mM KCl, 1.2 mM  $\text{NaH}_2\text{PO}_4$ , 1.4 mM  $\text{MgCl}_2$ , 10 mM HEPES, 6 mM glucose, 1 mM  $\text{CaCl}_2$  pH 7.4). The experiment was carried out in the dark and all solutions were kept on ice. Cells, plated and treated as previously described, were rinsed with PBS and 3 ml of the diluted Ro 19-8022 solution was added to each well. The plates were irradiated on ice at a distance of 33 cm from a 500 W halogen lamp, for 5 min. Control cells were incubated in the presence of PBS alone. After washing the cells with PBS to remove traces of Ro 19-8022, the Comet assay was performed as described above, with a small change: following lysis the slides were washed 3 times (5 min each) in cold enzyme reaction buffer (40 mM HEPES-KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8, 4 °C), blotted dry and incubated with the

repair enzyme (FPG), or buffer, for 30 min, at 37 °C. The amount of oxidized purines (FPG-sensitive sites) was then determined by subtracting the amount of strand breaks (samples incubated with buffer alone) to the total amount of breaks obtained after incubation with FPG.

### 2.6. In vitro base excision repair assay

The base excision repair (BER) assay measures the ability of a cell-free extract to recognize the damage in the DNA of substrate nucleoids and incise the DNA containing specific damage, in this case 8-oxoguanine (8-oxoGua). The increase in the amount of strand breaks produced reflects the DNA repair activity of the cell extract (Collins, 2004).

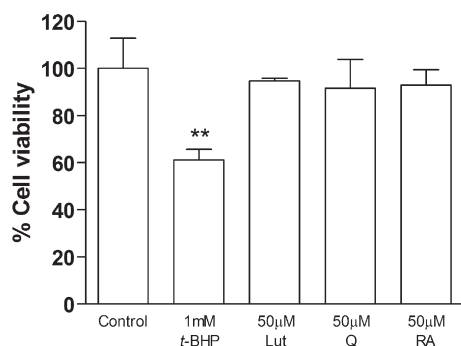
L929 fibroblasts were used as substrates. This allows an easier measurement of comets damage due to the greater size of these cells and their lack of a tendency to form aggregates when compared to PC12 cells. Briefly, L929 cells in a 75  $\text{cm}^2$  flask near confluence were treated with the photosensitizer Ro 19-8022 plus visible light (5 min irradiation on ice at 33 cm from a 500 W halogen lamp). Cells were then washed with PBS, trypsinized and collected in 5 ml of culture medium. After a centrifugation at 400 g for 5 min, at 4 °C, in a Sigma 2K15 microcentrifuge, the pellet was suspended in freezing medium (culture medium with 20% foetal bovine serum and 10% DMSO) at a density of  $3 \times 10^6$  cells/ml. Cells were then aliquoted and stored at  $-80$  °C.

For the extract preparation, PC12 cells, at a density of  $5 \times 10^6$  cells/ml, were incubated with the polyphenols for either 1 or 24 h. Cells were then washed with PBS, trypsinized and collected in PBS to microcentrifuge tubes. After centrifuging at 800 g for 5 min, at 4 °C, in a Sigma 2K15 microcentrifuge, the pellets were resuspended in ice cold PBS. Cells were divided into 1 ml aliquots and centrifuged at 14,000 g for 5 min, at 4 °C. Supernatants were discarded and the dry pellets flash frozen in liquid nitrogen, to be stored at  $-80$  °C. On the day of the experiment, one of these aliquots was thawed and the pellet resuspended in 65  $\mu\text{l}$  of lysis buffer (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, pH 7.8 with KOH, supplemented with 0.25% Triton X-100). The mixture was vortexed for 5 s, followed by a 5 min incubation on ice, and then centrifuged at 14,000 g for 5 min, at 4 °C. Finally, 55  $\mu\text{l}$  of the supernatant were removed and combined with 220  $\mu\text{l}$  of cold enzyme reaction buffer.

An aliquot of Ro- and light-treated substrate L929 cells was thawed, washed twice in cold PBS and spun for 5 min at 800 g, 4 °C. The pellets were suspended in 100  $\mu\text{l}$  of PBS and 35  $\mu\text{l}$  of this suspension was mixed with 1.5 ml of 1% low-melting point agarose at 37 °C. These cells were then embedded in agarose and the procedure then followed according to the standard Comet assay, with a slight modification: after lysis, the slides were washed three times (for 5 min each) in cold enzyme reaction buffer and incubated with 35  $\mu\text{l}$  of treated or non-treated extract for 20 min. Control slides incubated with either FPG (positive control) or buffer alone (negative control) were performed.

### 2.7. Quantification of rOGG1 and rAPE1 expression

Total RNA was isolated from PC12 cells treated in the same conditions as for the base excision repair assay using a Qiagen RNeasy total RNA isolation kit (Qiagen, USA), following standard protocol. RNA purity was confirmed by determining the OD260/OD280 nm absorption ratio. 1  $\mu\text{g}$  of total RNA was reversed transcribed with the Superscript Reverse Transcriptase III kit (Invitrogen, USA), by using 50 ng/ $\mu\text{l}$  of random hexamers and 10 mM of a dNTP mix according to the manufacturer's instructions. cDNA integrity was verified by gel electrophoresis after PCR amplification of Gapdh, using sequence-specific primers.



**Fig. 2.** Cell viability assessed by the MTT reduction test. PC12 cells were incubated overnight with increasing concentrations of the polyphenolic compounds. Control cells were incubated in the presence of 1% DMSO. Each bar represents the mean  $\pm$  S.E.M., considering the results obtained in at least three independent experiments. Cell damage induced by 1 mM *t*-BHP was used as a positive control. \*\* $P \leq 0.01$ , compared with the control.

PCR was performed using 18S specific primers (forward: 5'-AAG TCC CTG CCC TTT GTA CAC A-3'; reverse: 5'-GCC TCA CTA AAC CAT CCA ATC G-3') as an internal reference. Specific primer pairs for *r*OGG1 (forward: 5'-ACT TAT CAT GGC TTC CCA AAC C-3'; reverse: 5'-CAA CTT CCT CAG GTG GGT CTC T-3') and *r*APE1 (forward: 5'-GCG GCA GCG GAA GAC-3'; reverse: 5'-GCC TCC TTC TCA GTT TTC TTT GCT-3') were described in Englander and Ma (2006). Primers were manufactured by STAB-VIDA, Portugal.

Real-time RT-PCR was performed in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, USA) using 1  $\mu$ l of the cDNA preparation, which was added to a reaction mixture containing 12.5  $\mu$ l Power SYBR Green master mix (Applied Biosciences, Cheshire, UK), 1  $\mu$ l of each primer (25 pmol/ $\mu$ l) and autoclaved water to a final

volume of 25  $\mu$ l per well. The plates were covered, centrifuged and placed in the thermal cycler. The PCR conditions used were: 50  $^{\circ}$ C for 2 min, 95  $^{\circ}$ C for 10 min and 40 cycles (95  $^{\circ}$ C, 15 s; 60  $^{\circ}$ C, 60 s).

The expression of OGG1 and APE1 mRNA in samples was determined from a standard curve constructed from serial dilutions of cDNA obtained from unstimulated PC12 cells. Target genes' transcript levels were all normalized to 18S mRNA levels. The average of at least two replicates for each of three independent experiments was used.

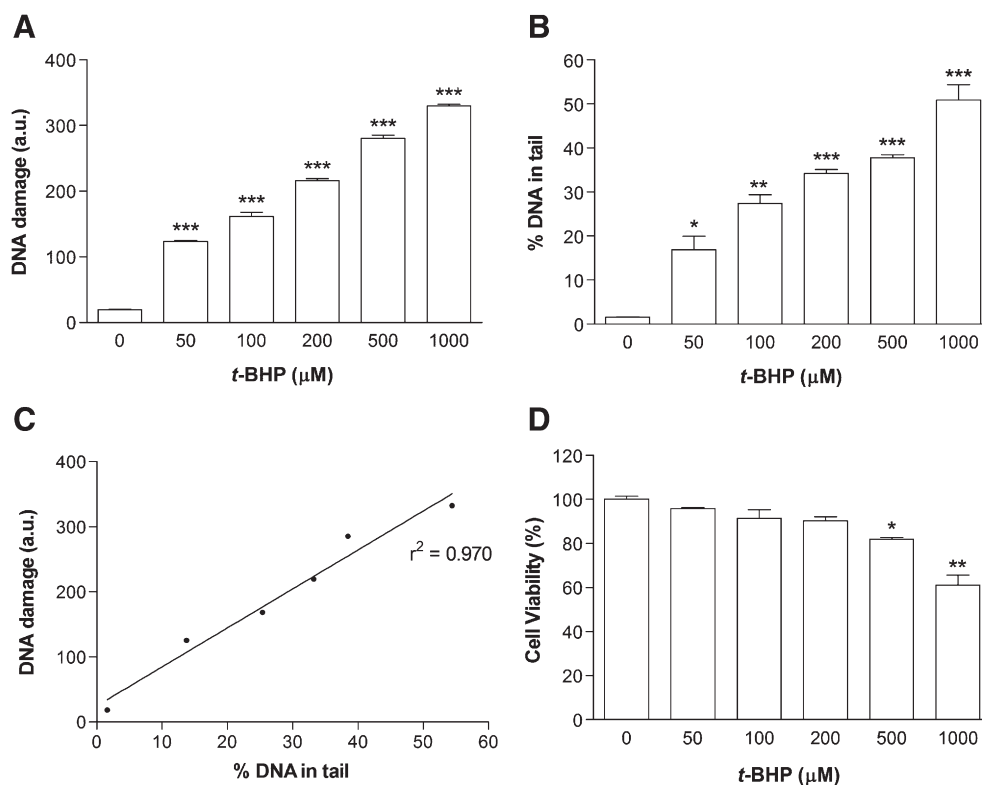
## 2.8. Statistical analysis

Data are expressed as the mean  $\pm$  S.E.M., of the indicated number of experiments. The significance of the differences between the means observed was evaluated using the unpaired two-tailed Student's *t*-test. A difference of  $P \leq 0.05$  was considered significant.

## 3. Results

### 3.1. Polyphenols toxicity to PC12 cells

The beneficial effects of polyphenolic compounds like the ones herein tested in cells under oxidative stress are well recognized (Sasaki et al., 2002; Horvathova et al., 2005; Iuvone et al., 2006). Nevertheless, in specific conditions, some of them can also induce harmful effects. For example, quercetin, was described as becoming toxic as a result of its own protective activity (Leung et al., 2005; Hur et al., 2007; Boots et al., 2007). We therefore evaluated the cytotoxicity of quercetin, luteolin and rosmarinic acid in our biological model. As depicted in Fig. 2, for the concentrations tested, none of the compounds showed a statistical significant decrease in cell viability, even after an overnight incubation.



**Fig. 3.** *t*-BHP-induced damage in PC12 cells. Cells were incubated with different concentrations of *t*-BHP for 1 h and DNA damage assessed by the Comet assay. Comets were quantified either by visual scoring (A) or by computer-assisted image analysis (B). C) Correlation coefficient between the semi-quantitative method and the computer assisted parameter. D) Cell viability assessed by MTT reduction test. Each bar represents the mean  $\pm$  S.E.M., for at least three independent experiments. \* $P \leq 0.05$ , \*\* $P \leq 0.01$  and \*\*\* $P \leq 0.001$ , compared with the control.



### 3.2. Determination of *t*-BHP-induced damage to PC12 cells

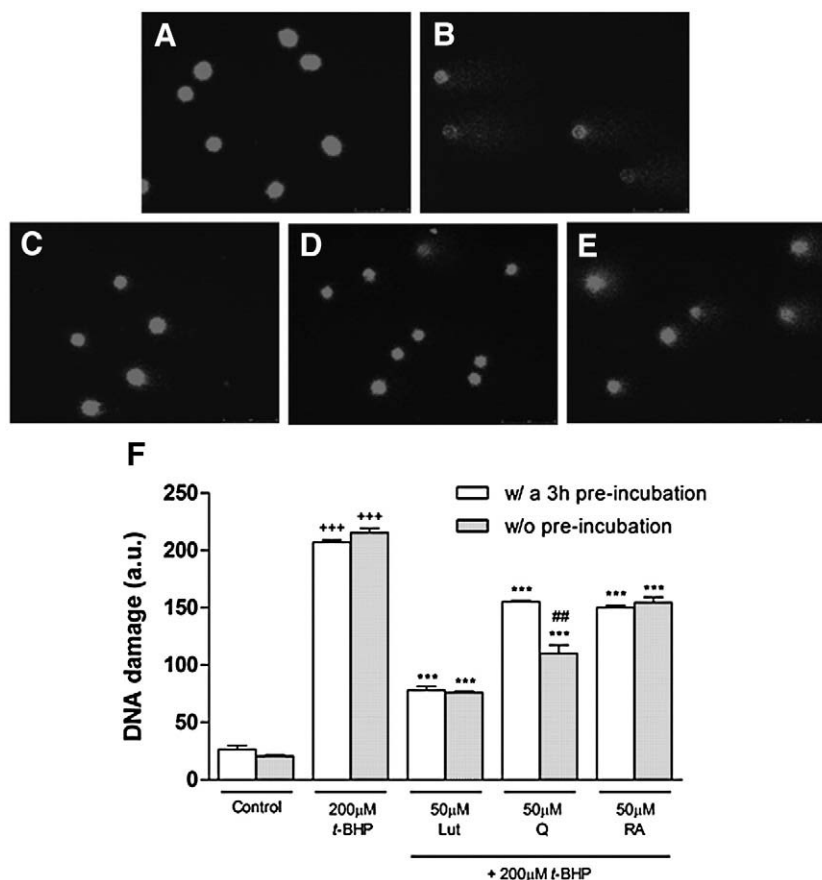
The extent of oxidative DNA damage was evaluated by the Comet assay, by incubating PC12 cells for 1 h with increasing concentrations of the thiol-oxidizing agent, *t*-BHP, a widely accepted inducer of oxidative stress (Ahmed-Choudhury et al., 1998; Palomba et al., 2001; Pias and Aw, 2002). The dose–response curves to *t*-BHP on DNA damage (Fig. 3A and B) obtained by comet quantification assessed both by visual scoring (A) and computer-assisted image analysis (B), indicate a concentration-dependent effect in DNA damage. The results showed a good correlation between the semi-quantitative method of visual scoring and the % of DNA in the tail assessed by computerized image analysis (Fig. 3C), which is in accordance with other authors (Collins et al., 1997). Despite a considerable amount of DNA damage being observed for concentrations above 200  $\mu$ M, cell viability (assessed by the MTT assay) was significantly decreased at concentrations of 500 and 1000  $\mu$ M (Fig. 3D). Therefore, for subsequent induction of oxidative DNA damage, PC12 cells were incubated with 200  $\mu$ M *t*-BHP for 1 h.

### 3.3. Evaluation of the protective effect of the compounds against DNA strand breaks formation

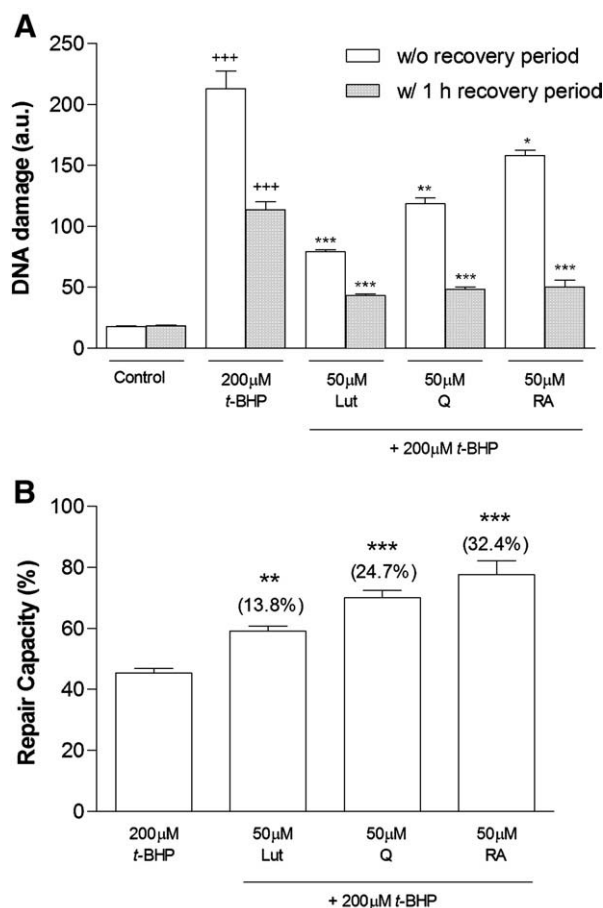
The interaction between reactive oxygen species and DNA can lead to the oxidation of this biomolecule, resulting in several types of oxidative DNA damage, including strand breaks and oxidized bases (Saitoh et al., 2001). We firstly studied the protective effects of the compounds against the formation of strand breaks, induced by *t*-BHP.

The polyphenolic compounds were either pre-incubated for a period of 3 h, or incubated simultaneously with the oxidative stimulus. As it can be observed in Fig. 4, all the compounds significantly decreased the *t*-BHP-induced formation of DNA strand breaks. Among them, luteolin showed the highest protective effect, with a reduction in DNA damage of about 71% for either incubation conditions. No differences could be observed between pre-incubating or not the cells with the luteolin or rosmarinic acid. For quercetin, the protective effect was significantly increased when added simultaneously with *t*-BHP (28.8 and 54.1% with or without pre-incubation, respectively). The results relative to the simultaneous incubation of the cells with the compounds and *t*-BHP are clearly demonstrated by the comets representative images for each condition (Fig. 4, A–E).

We then investigated the ability of these compounds in the stimulation of strand breaks repair. In this way, after cell incubation in the presence of the polyphenolic compounds and 200  $\mu$ M *t*-BHP for 1 h, culture media was replaced and cells given a 1 h recovery period, to allow the repair of DNA damage. After this recovery period, and in the presence of the deleterious stimulus alone, cells had repaired 45.3% of strand breaks (Fig. 5). When cells were co-incubated with the polyphenols, the strand breaks repair capacity was significantly increased. There seems to be an inverse correlation between the compounds capacity to protect the cells against the formation of strand breaks and their ability to increase the cells' repair capacity of this kind of damage. In fact, despite having a weaker protective effect against the formation of strand breaks, rosmarinic acid induced the highest increase in repair capacity (32.4% above cells own repair), followed by quercetin and luteolin, which increased repair capacity by



**Fig. 4.** Compounds protection against *t*-BHP-induced formation of DNA strand breaks, evaluated by the Comet assay. A–E) representative images of Comets from control, 200  $\mu$ M *t*-BHP, 50  $\mu$ M luteolin, 50  $\mu$ M quercetin and 50  $\mu$ M rosmarinic acid (without pre-incubation), respectively. F) Quantification of DNA damage by visual scoring. PC12 cells were incubated for 1 h in the presence of 200  $\mu$ M *t*-BHP. Polyphenolic compounds were added to the cells either at the same time or 3 h prior to the addition of the oxidative stimulus. Each bar represents the mean  $\pm$  S.E.M for at least three independent experiments. \*\*\* $P \leq 0.001$ , compared to respective control cells; \*\*\*\* $P \leq 0.001$ , compared to 200  $\mu$ M *t*-BHP (for each respective incubation condition); ## $P \leq 0.01$ , compared with the same compound, after a 3 h pre-incubation.

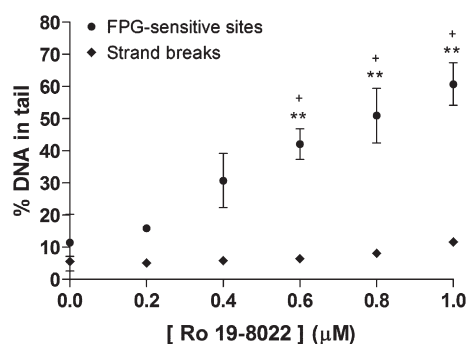


**Fig. 5.** Effects of the compounds on DNA strand breaks repair. A) After 1 h of cells' simultaneous incubation with the compounds and 200 μM *t*-BHP, cell culture media was replaced and DNA damage evaluated 1 h later. Comets were visually scored and results expressed in percentage of damage relatively to the maximum obtained before the recovery period. B) Repair capacity was calculated using the formula presented in the Materials and methods section. Values in brackets represent the increase in repair capacity. For each bar is represented the mean±S.E.M for at least three independent experiments. \*\*\* $P \leq 0.001$ , compared to respective control cells; \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , compared to 200 μM *t*-BHP (for each respective incubation condition).

24.7% and 13.8%, respectively. These results reflect the ability of these compounds to protect against DNA strand breaks formation and, more importantly, to enhance the repair of this type of damage, suggesting an involvement on specific mechanisms of DNA repair.

### 3.4. Effects of the polyphenolic compounds on base oxidation

In addition to formation of strand breaks, structure modifications at the level of nucleotidic bases can occur as a consequence of oxidative stress. Oxidation of nucleotidic bases is likely to be as important as DNA strand breaks to overall cellular function and survival (Bjelland and Seeberg, 2003). We therefore investigated the ability of the polyphenols herein studied to help cells overcome the formation of oxidized bases. Since *t*-BHP induced a great amount of strand breaks along with bases oxidation, making it difficult to distinguish between these two types of damage, we used Ro 19-8022, a photosensitizer compound that induces only the accumulation of oxidized bases, namely 8-oxoGua, without the induction of a significant amount of strand breaks (Angelis et al., 1999; Gedik et al., 2002). Indeed, treatment of PC12 cells with increasing concentrations of Ro 19-8022 (Fig. 6) resulted in a gradual increase in the percentage of DNA in the comet tails. From these results, we selected the 0.6 μM Ro 19-8022 concentration as the most adequate for subsequent experiments, since a significant increase in DNA damage could be

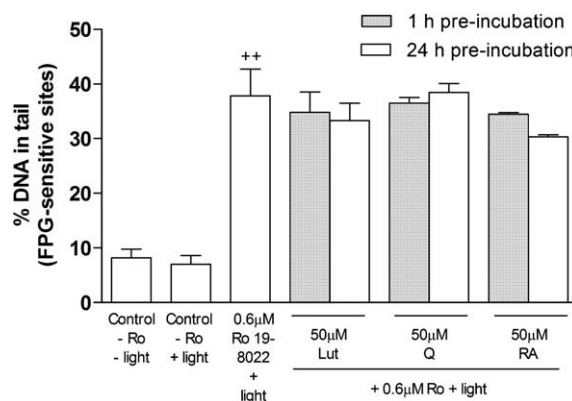


**Fig. 6.** Dose-response curve for cells treatment with Ro 19-8022. PC12 cells were incubated for 5 min, on ice and under a 500 W halogen lamp, in the presence of increasing concentrations of Ro 19-8022. The level of oxidized purines was determined through sample incubation with FPG, for 30 min. FPG-sensitive sites were obtained by subtracting the amount of strand breaks alone to the amount of breaks obtained after incubation with FPG. Percentage of damage in the comet tail was quantified by computer-assisted image analysis. For each condition is represented the mean±S.E.M for at least three independent experiments. \* $P \leq 0.05$ , compared to cells in the absence of Ro 19-8022; \*\* $P \leq 0.01$ , relatively to strand breaks for the same condition.

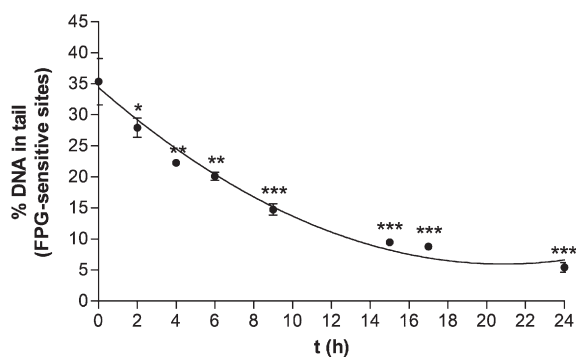
observed, with few class 4 comets generated. This indicates that the assay had not reached a saturation point, which must be avoided, since it could lead to underestimation of enzyme-sensitive sites (Gedik et al., 2002).

The polyphenolic compounds effects were then evaluated on the formation of oxidized bases induced by 0.6 μM Ro 19-8022. Two pre-incubation periods, 1 h and 24 h, were tested. Results in Fig. 7 show that none of the compounds significantly reduced the amount of oxidized purines, independently of the pre-incubation period used.

We also investigated the effects of the polyphenolic compounds after a recovery period after the incubation with Ro 19-8022, since time can be decisive for the observation of a possible protective effect. In this way, after Ro 19-8022 treatment, culture media was replaced and cells allowed to recover for a period of 6 h. The selection of this time period was based on results from Fig. 8, which show that, after a 6 h recovery, cells had already repaired about 51% of oxidized bases and the assay had not reached saturation. These results are in agreement with previous reports by other authors using different cell lines, and can be attributed to the longer period of time required by oxidized bases to be repaired, when compared to strand breaks (Collins and Horvathova, 2001).

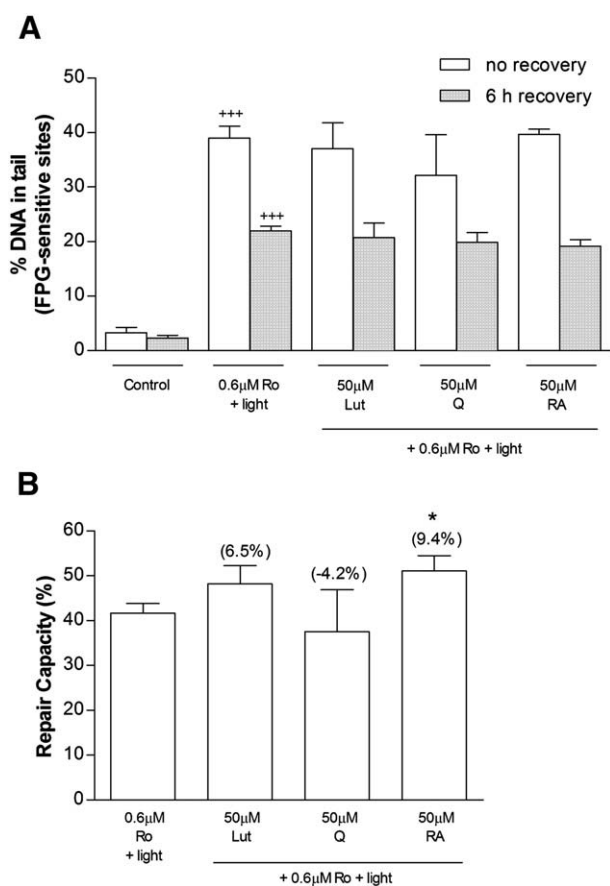


**Fig. 7.** Effect of the polyphenolic compounds on Ro 19-8022-induced purines oxidation. PC12 cells were either incubated for 1 h or 24 h, prior to the treatment with 0.6 μM Ro 19-8022 plus light. The level of oxidized purines was determined through sample incubation with FPG, for 30 min. Percentage of damage in the comet tail was quantified by computer-assisted image analysis. Each bar represents the mean±S.E.M for at least three independent experiments. \*\* $P \leq 0.01$ , compared to cells in the absence of Ro 19-8022.

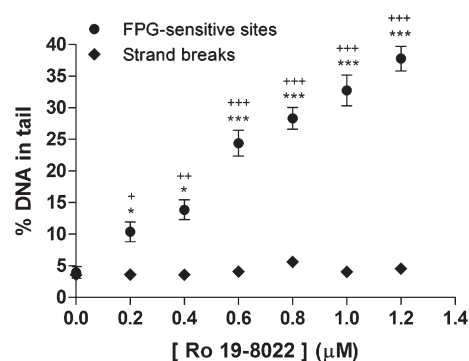


**Fig. 8.** DNA repair of Ro-induced oxidized purines. PC12 cells were treated with Ro 19-8022 plus light for 5 min, on ice. Culture media was then replaced to allow damage repair. At defined time-points, cells were collected and assayed for the level of oxidized purines, by incubating samples with FPG. Percentage of damage in the comet tails was quantified by computer-assisted image analysis. For each condition is represented the mean  $\pm$  S.E.M. for at least three independent experiments. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$  compared to cells in the absence of Ro 19-8022.

As presented in Fig. 9A, after a 6 h recovery period following a 24 h incubation of the cells in the presence of the polyphenolic compounds, the levels of DNA in the comet tails was not significantly altered by the presence of the polyphenols. Nevertheless, after cal-



**Fig. 9.** Effects of the compounds on purine oxidation repair. A) After a 24 h incubation in the presence of the compounds, cells were treated with Ro 19-8022 plus light for 5 min, on ice. Culture media was then replaced and DNA damage evaluated by the Comet assay 6 h later. Percentage of damage in the comet tail was quantified by computer-assisted image analysis. B) Repair capacity calculated according to the formula presented in Materials and methods. Values in brackets represent the increase in repair capacity. For each bar is represented the mean  $\pm$  S.E.M. for at least three independent experiments. \*\*\* $P \leq 0.001$ , compared to respective control cells; \* $P \leq 0.05$ , compared to 0.6  $\mu$ M Ro 19-8022 + light.



**Fig. 10.** Dose-response curve of Ro 19-8022-induced DNA damage. L929 cells were incubated in the same conditions indicated in Fig. 6. Levels of oxidized purines were determined and quantified as previously described. For each condition is represented the mean  $\pm$  S.E.M. for at least three independent experiments. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$  compared to cells in the absence of Ro 19-8022; \* $P \leq 0.05$ , \*\*\* $P \leq 0.001$ , relatively to strand breaks for the same condition.

culating the repair capacity induced by each compound, we observed that rosmarinic acid significantly increased the cells capacity to repair oxidized purines in about 9.4% (Fig. 9B). On the other hand, neither luteolin nor quercetin were able to significantly increase the cells' intrinsic repair capacity, although for luteolin there seems to exist a trend to do so. The lack of effect obtained for quercetin had been previously reported in lymphocytes, using hydrogen peroxide as an inducer of base oxidation (Duthie and Dobson, 1999). Our results clearly indicate that, among the polyphenolic compounds tested, only rosmarinic acid seems to affect the repair of oxidized bases.

### 3.5. In vitro base excision repair assay

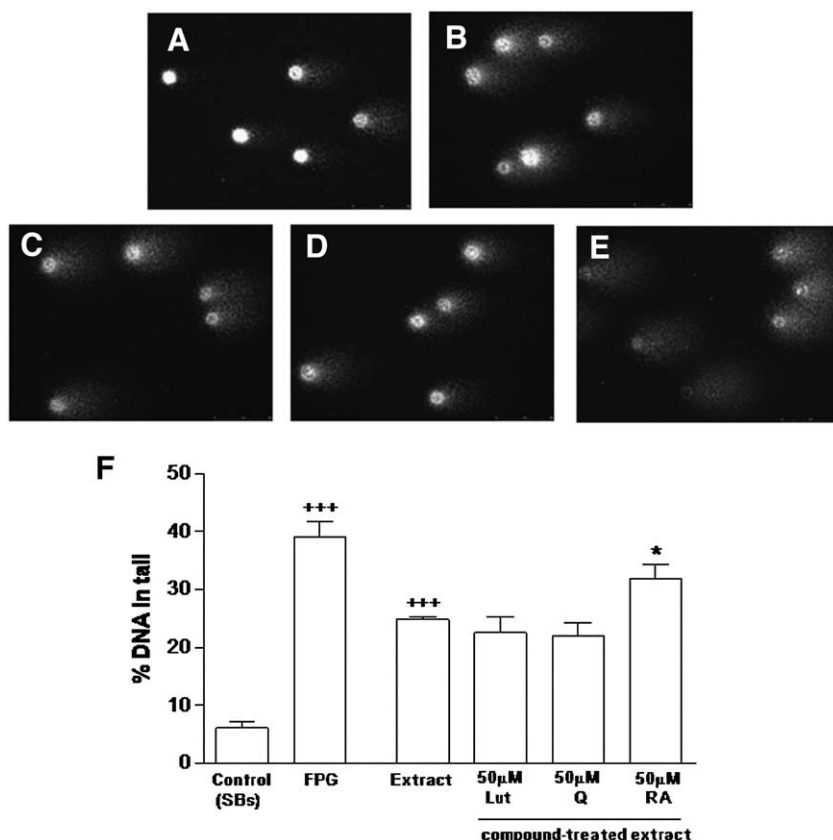
The capacity for incision activity by repair enzymes at oxidized purines in DNA was monitored in cell-free extracts, obtained from PC12 cells previously incubated in the presence of the polyphenols for 24 h. Damage-containing substrates were obtained by treating L929 cells with 1  $\mu$ M Ro 19-8022, for 5 min on ice, under a 500 W halogen lamp. This concentration was selected based on a dose-response curve of DNA damage against Ro 19-8022 (Fig. 10).

Results in Fig. 11 show that after a 20 min incubation of the substrate with a cell-free extract, L929 suffer an increase in the number of DNA breaks relatively to the control ( $24.84 \pm 0.47\%$  vs  $6.12 \pm 0.97\%$ ,  $P \leq 0.001$ ), as previously observed by Collins et al. (2003). Since substrate cells were treated with Ro 19-8022 and light, these breaks reflect only the amount of oxidized nucleotidic bases. It is also evident in the same figure that only rosmarinic acid led to an improvement of PC12 cells' capacity to repair this kind of damage, as demonstrated by an increase of 6.96% of DNA in the tail for the rosmarinic acid-treated extract. This is equivalent to a 37.1% increase in DNA repair capacity relatively to the non-treated extract. In the presence of extracts treated with either luteolin or quercetin, DNA repair was unaffected. The increase in incision activity induced by the rosmarinic acid-treated extract, as well as the lack of effect observed for luteolin and quercetin, are clearly demonstrated in the representative images of BER assay (Fig. 11A–E).

These data suggest a possible involvement of rosmarinic acid in the regulation of gene expression of DNA repair enzymes.

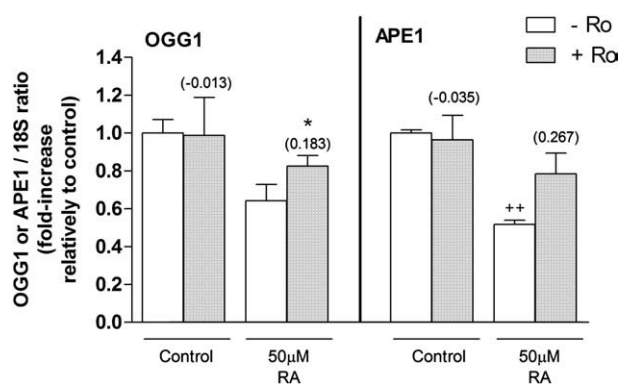
### 3.6. Expression of DNA repair genes

We tried to elucidate the molecular mechanisms underlying the results observed with rosmarinic acid, namely by investigating a direct effect of this compound on the expression of the DNA repair genes OGG1 and APE1, which play an important role in DNA repair,



**Fig. 11.** Effect of the compounds on the *in vitro* BER assay. A–E) representative images of Comets (purines only) from control (buffer alone), extract from non-treated cells, extract from 50  $\mu$ M luteolin-treated cells, extract from 50  $\mu$ M quercetin-treated cells and extract from 50  $\mu$ M rosmarinic acid-treated cells, respectively. F) Quantification of the percentage of DNA in the comet tails was done after treatment of damage-containing substrate cells with a cell-free extract obtained from PC12 cells incubated with the polyphenols for 24 h. The repair enzyme FPG was used as a positive control. Levels of oxidized purines were determined and quantified as previously described. For each condition is represented the mean  $\pm$  S.E.M for at least three independent experiments. \*\*\* $P \leq 0.001$  compared to control cells; \* $P \leq 0.05$ , relatively to non treated extract. SBs – Strand Breaks.

using *real time* RT-PCR. Treatment of PC12 cells with rosmarinic acid alone results in a significant decrease in the expression of APE1, (Fig. 12, white bars). However, in the presence of Ro 19-8022, rosmarinic acid seems to significantly enhance the expression of the repair gene OGG1. Such results confirm the action of rosmarinic acid at the level of DNA repair, which is discussed further on.



**Fig. 12.** Effects of rosmarinic acid on the expression of repair genes OGG1 and APE1. PC12 cells were incubated for 24 h in the presence of rosmarinic acid, prior to the exposure to Ro 19-8022 plus visible light. Levels of genes expression was determined by *real time* RT-PCR and results expressed as the increase in the expression of each gene relatively to the control. Values in brackets represent the difference in the fold-increase expression between Ro-treated and non-treated conditions. Each bar represents the mean  $\pm$  S.E.M for at least two independent experiments. \*\* $P \leq 0.01$ , compared to control cells in the absence of Ro 19-8022; \* $P \leq 0.05$ , compared to cells treated with rosmarinic acid alone.

#### 4. Discussion

Oxidative DNA damage is generally regarded as a dynamic steady-state: an equilibrium is maintained between input of damage (*i.e.* endogenous and/or exogenous free radicals attack), which can be attenuated by antioxidant defences, and output from this damage (*i.e.* DNA repair). A change in input or output will lead to an increase or decrease in the level of damage, until a new equilibrium is attained. The implication is that DNA repair, in normal physiological conditions, maintains oxidative damage at a level that is tolerable in terms of genetic stability (Tomasetti et al., 2001; Collins and Gaivão, 2007).

In this work, three polyphenolic compounds (luteolin, quercetin and rosmarinic acid) were investigated for their protective effects on oxidative DNA damage in a neuronal cell model, with a particular focus on the mechanisms by which they may be acting. Their antioxidant properties have already been well characterized in the literature (Noroozi et al., 1998; Petersen and Simmonds, 2003) and, in addition, some authors have described their protective effects against oxidative DNA damage induced in cancer cell lines, in a clear association with their anti-carcinogenic properties (Duthie and Dobson, 1999; Lima et al., 2006). In neuronal cells, it is described the cumulative effect of DNA damage in human brain over time (especially in mitochondrial DNA), which is supposed to play a critical role in aging and in the pathogenesis of several neurodegenerative diseases (Bjelland and Seeberg, 2003; Coppede et al., 2007; Fishel et al., 2007).

To date, no studies have reported the effects of these polyphenols on oxidative DNA damage in neuronal models. Additionally, it remains unknown whether these compounds exert their protective effects against oxidative DNA damage by enhancing DNA repair or simply by preventing the oxidation of DNA due to their antioxidant properties.



Based on the fact that, in our cell model, a 50  $\mu\text{M}$  concentration of the polyphenolic compounds did not present any toxicity to cells, and after selecting the best conditions to obtain a significant amount of DNA damage without inducing a significant decrease in cell viability, we proceeded to investigate the compounds protection against *t*-BHP-induced oxidative DNA damage. It should be noted that the use of such concentrations has been also reported in other works with neuronal cell models (Sasaki et al., 2003; Park et al., 2007; Okawara et al., 2007) and for some authors were considered low (Horvathova et al., 2005). As expected from previous reports, regarding the protective effects of these polyphenolic compounds on oxidative DNA damage (Noroozi et al., 1998; Duthie and Dobson, 1999; Lima et al., 2006), all of them were also able to decrease *t*-BHP-induced DNA strand breaks formation in our cell model. Among them, luteolin proved to be the most efficient one, which is in accordance with previous studies reporting the high antioxidant activity for this compound, observed in different cells, such as skin fibroblasts or macrophages (Filipe et al., 2005; Harris et al., 2006). Although no differences in protection were observed when luteolin or rosmarinic acid were added simultaneously or 3 h prior to the addition of *t*-BHP, quercetin presented an ameliorated effect when simply co-incubated with the deleterious stimulus. A similar effect has been reported in human lymphocytes and was attributed to the metabolism of this flavonoid into a less active compound (Duthie and Dobson, 1999).

The ability of the compounds to increase strand breaks repair varies in the inverse order of that observed for the prevention of strand breaks formation. In fact, although all the compounds tested were able to enhance the repair of strand breaks in PC12 cells, rosmarinic acid induced the highest repair capacity for this kind of damage. Since both luteolin and quercetin are known to possess high antiradical scavenging activities (Noroozi et al., 1998; Choi et al., 2003; Horvathova et al., 2005), and based on the fact that most of their protective effects against DNA damage occurred during the 1 h incubation with *t*-BHP, we are led to believe that these effects are mainly due to their antioxidant properties. In addition, features such as their high lipophilicities (Areias et al., 2001) and abilities to chelate metal ions (Mira et al., 2002) may also be responsible for their protective effects. Rosmarinic acid, by its turn, has a weaker reactive oxygen species scavenging activity and a lower lipophilicity when compared to luteolin and quercetin, thus the results obtained suggest its involvement in the intracellular pathways related to DNA repair.

The hypothesis that rosmarinic acid could be acting on those intracellular pathways, in opposition to the more direct effect of luteolin and quercetin, was further studied by investigating the involvement of these polyphenolic compounds on the protection against base oxidation. It should be taken into account that the effect of antioxidants on recovery from oxidative DNA damage may be justified by at least two different explanations: 1) by stimulating the activity of repair enzymes or 2) through a direct protection against oxidation (Tomasetti et al., 2001). In the conditions tested, none of the polyphenols was able to decrease the amount of oxidized purines induced by the photosensitizer compound, independently of the time used in the pre-treatment. Nevertheless, rosmarinic acid significantly increased the PC12 cell capacity to repair oxidized purines. For luteolin, a trend to increase the repair of oxidized purines was observed, though this increase was not statistically significant.

Since Ro 19-8022 can induce a great amount of DNA damage in a short period of time, the antioxidant properties of the polyphenols tested may not be sufficient to exert a protective effect against DNA damage induced by this agent. Instead, a protective effect in this case, to be detected, may be more easily attributed to an involvement of a compound in the mechanisms responsible for Ro 19-8022 toxicity. Considering this, the results obtained are indicative that only rosmarinic acid may be acting on the intracellular pathways leading to repair of DNA damage.

In order to better re-create *in vitro* the conditions for DNA repair occurring *in vivo*, an alternative approach for the assessment of repair capacity of a sample extract, provided with a DNA substrate carrying

specific type of damage, was used (Tomasetti et al., 2001). We observed that only rosmarinic acid was able to increase the PC12 cells' capacity to repair oxidized bases, as indicated by the greater amount of breaks formed in the presence of extract pre-treated with this compound, relatively to the non-treated extract. This effect on DNA repair appears to be mediated through change in gene expression, namely an increase in OGG1 mRNA levels. However, in the presence of rosmarinic acid alone the levels of repair genes expression decreased. This might be explained considering the dynamic steady state of oxidative DNA damage, previously referred. Based on this theory (Collins and Gaivão, 2007) we may assume that, in the presence of rosmarinic acid, basal damage input to DNA induced by the deleterious stimulus will be lower than in the absence of the compound. In this situation, a smaller amount of oxidized nucleotidic bases might be expected. So, repair enzymes might not be recruited to a great extent, which would explain the lower expression of repair genes. In addition, the lack of a significant effect of rosmarinic acid on APE1 expression may be attributed to a later recruitment of this enzyme during the repair process, as also described elsewhere (Hill et al., 2001). It should be further noted that DNA repair enzymes, such as OGG1, are considered housekeeping genes (Loft and Moller, 2006). In this way, an effect at the level of base oxidation repair, if present, should be subtle, since the intracellular pathways that are involved in the repair of oxidative DNA damage are vital to cells and are therefore tightly regulated. This fact also justifies the small variation obtained in expression of these genes with or without the deleterious stimulus.

Extracts pre-treated with either luteolin or quercetin were unable to increase the DNA repair capacity, confirming that these compounds cannot act against the oxidation of nucleotidic bases induced by Ro 19-8022.

In this study, we have shown some of the underlying mechanisms associated to the protective effects on oxidative DNA damage in PC12 cells for three polyphenolic compounds: luteolin, quercetin and rosmarinic acid. While the action of both luteolin and quercetin seems to be associated with a direct effect on reactive oxygen species scavenging, with major implications on the prevention of *t*-BHP-induced strand breakage (in accordance with results reported in other cell models), rosmarinic acid may rather exert an indirect effect, by mediating intracellular mechanisms responsible for DNA repair. However, it should be taken into account that this study intended to be the first step in the evaluation of the protective effects of these polyphenols on oxidative DNA damage induced to neuronal cells. Before any correlations can be made between the protection observed *in vitro*, in this cell model, and what happens *in vivo*, bioavailability studies should be performed. Some polyphenols do not pass easily through the blood brain barrier and therefore are likely present, in the central nervous system, at low concentrations. This has been reported for both quercetin and rosmarinic acid (Youdim et al., 2004; Li et al., 2007) but not for luteolin which seems to have a good ability to cross the barrier (Hendriks et al., 2004). Nevertheless, a study with quercetin, showed that a combination of this flavonoid with phospholipids like lecithin is a way of increasing its ability to cross the blood brain barrier and exert its protective effect in the brain (Dajas et al., 2003). So a role for these compounds in neuroprotection should not be ruled out.

In conclusion, our results strongly suggest that the polyphenols tested, which are present in the human diet, and in particular rosmarinic acid, should be considered as valuable protectors against oxidative stress-induced DNA damage that commonly occurs in several pathological conditions, namely in neurodegenerative diseases.

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