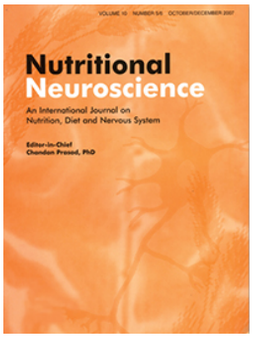

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Nutritional Neuroscience

An International Journal on Nutrition, Diet and Nervous System

ISSN: 1028-415X (Print) 1476-8305 (Online) Journal homepage: http://www.tandfonline.com/loi/ynns20

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To cite this article: Maria Elisabetta Clementi, Giovambattista Pani, Beatrice Sampaolese & Giuseppe Tringali (2017): Punicalagin reduces H_2O_2-induced cytotoxicity and apoptosis in PC12 cells by modulating the levels of reactive oxygen species, Nutritional Neuroscience, DOI: 10.1080/1028415X.2017.1306935

To link to this article: http://dx.doi.org/10.1080/1028415X.2017.1306935

Published online: 09 Apr 2017.

Punicalagin reduces H_2O_2-induced cytotoxicity and apoptosis in PC12 cells by modulating the levels of reactive oxygen species

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Background: Oxidative stress has long been linked to neuronal cell death in many neurodegenerative diseases. Antioxidant conventional supplements are poorly effective in preventing neuronal damage caused by oxidative stress due to their inability to cross the blood brain barrier. Hence the use of molecules extracted from plants and fruits such as phenolics, flavonoids, and terpenoids compounds constitute a new wave of antioxidant therapies to defend against free radicals.
Objective: In this study we examined the effects of punicalagin, a ellagitannin isolated from the pomegranate juice, on a rat adrenal pheochromocytoma cell line, treated with hydrogen peroxide, evaluating the viability, oxidation potential, mitochondrial function, and eventual apoptosis.
Methods: This study was performed on PC12 cells pretreated with punicalagin (0.5, 1, 5, 10 e 20 μM) 24 hours before of the damage by hydrogen peroxide (H_2O_2). H_2O_2 concentration (300 μM) used in our study was determined by preliminary experiments of time course. The cell viability and ROS production were evaluated by MTS assay and cytofluorometry assays, respectively. Subsequently, the number of apoptotic-positive cells and mitochondrial transmembrane potential, were measured by flow cytometry, in the same experimental paradigm. Finally, the expression of Bax and enzymatic activity of Caspase 3, some of the principle actors of programmed cell death, were investigated by semiquantitative PCR and utilizing a colorimetric assay kit, respectively.
Results: We found that pretreatment with punicalagin protected the cells from H_2O_2-induced damage. In particular, the protective effect seemed to be correlated with a control both in radical oxygen species production and in mitochondrial functions. In fact the cells treated with H_2O_2 showed an altered mitochondrial membrane integrity while the pretreatment with punicalagin retained both the cellular viability and the mitochondrial membrane potential similar to the control. Furthermore, the punicalagin, modulated the apoptotic cascade triggered reducing Bax gene expression and Caspase 3 activity.
Discussion: Results of the present study demonstrated a neuroprotective effect of punicalagin on H_2O_2-induced PC12 cell death, including mitochondria damage and expression of apoptotic gene Bax; therefore we hypothesize a possible prevent role for this molecule in neurodegenerative diseases related to oxidative stress.

Keywords: Oxidative stress, Punicalagin, Neurodegeneration, Apoptosis, Mitochondrial dysfunction

Introduction

It has been recently assessed the pomegranate juice's ability to protect tissues and cells against stress-induced damage, with the idea that this dietary supplement may serve as a therapeutic agent.[1,2] Pomegranate possesses high amounts of ellagitannins such as punicalagin, punicalin, gallagic acid, ellagic acid and EA-glycosides[3]; in particular the punicalagin has been recently identified as a new anti-inflammatory, antiproliferative, hepatoprotective, and antigenotoxic molecule.[4–6]

The concentration of hydrogen peroxide (H_2O_2) in healthy individuals is normally low; however, it is frequently used as experimental model to study cellular

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DOI 10.1080/1028415X.2017.1306935

damage by oxidative stress and to evaluate the antioxidant effects of potential new therapeutic substances. Furthermore, oxidative stress is considered as the basis of some neurodegenerative disorders such as Alzheimer and Parkinson's disease, cerebral ischemia, and other cerebral pathologies in which are produced high amounts of reactive oxygen species (ROS).[7–9] Once ROS are generated as byproducts of cellular respiration, it is thought that mitochondria are the primary target of oxidative damage. Antioxidants can inhibit ROS formation in cells, and increase cellular defenses by up-regulating antioxidant gene transcription.[10,11]

Over the years, an accumulation of epidemiological data indicate the importance of a balanced diet rich in antioxidants to maintain proper cognitive functions and to prevent or delay neurodegeneration.[12]

Within this framework, in the present study we investigated the antioxidant effects of the pre-treatment with punicalagin on a pheochromocytoma cell line (PC12), after H_2O_2-induced oxidative damage, by assessing cell viability, ROS levels and the function mitochondrial.[13] Moreover, since mitochondria play a critical role in cell apoptosis regulation, we examined also the gene expression of Bax and activity of Caspase 3, two of the main actors of programmed cell death.

Methods

Cell line and treatments

PC12 cells (a clonal line of rat pheochromocytoma) were cultured in a 5% CO_2 atmosphere at 37°C in RPMI with HEPES 10 mM, glucose 1.0 g/l, $NaHCO_3$ 3.7 g/l, penicillin 100 units/ml, streptomycin 100 µg/ml, 10% Fetal Calf Serum, and 15% Horse Serum. Once grown until 85% confluence the cells were subcultured at an appropriate density according to each experimental procedures.

Punicalagin powder (Sigma Aldrich Co.-St. Louis, MO, USA) was dissolved in a 10 mM stock solution phosphate buffer.

Direct toxicity study

For determination of viability, PC12 cells were plated in 96-well plates at a density of 10 000 cells/well and incubated for 24 hours, with different concentrations (50, 100, 300, and 500 µM) of H_2O_2. Cell survival was evaluated by the 3-[(4,5-dimethylthiazol-2-yl)-5,3-carboxymethoxyphenyl]-2-(4-sulfophenyl)-2H tetrazolium, inner salt reduction assay. The MTS assay (Promega srl – Padova – Italy) is a sensitive measurement of the normal metabolic status of cells, which reflects early cellular redox changes. The intracellular soluble formazan produced by cellular reduction of the MTS was determined by recording the absorbance of 96-well plate using the automatic microplate photometer at a wavelength of 490 nm. Cell viability was expressed as a percentage of surviving cell. Successively, to determine the effective concentration of punicalagin, the cells were preincubated with punicalagin at different concentration (0.5, 1, 5, 10, and 20 µM) for 24 hours and then treated with H_2O_2 300 µM. At the light to these results, our successive investigations were performed pretreating PC12 cells with punicalagin 10 µM for 24 hours and subsequently with hydrogen peroxide 300 µM. The morphological features of PC12 cells exposed to different treatments were analyzed and photographed by phase-contrast microscopy 40×.

Measurement of cellular generation of reactive oxygen species

The detection of ROS was performed in 96-well microplate with 25 000 cells/well using 2′,7′-dichlorofluorescein diacetate (DCFDA) – Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, Cambridge, UK). Briefly, after 24 hours preconditioning with different concentrations (0.5, 1, 5, 10, and 20 µM) of Punigalagin, the cells were treated with H_2O_2 300 µM. After 24 hours the cells were washed with phosphate buffer three times and then treated with DCFDA which is initially non-fluorescent and is converted by oxidation to the fluorescent molecular DCF. DCF was then quantified using a CytoFluor Multi-well Plate Reader, with 485 nm excitation and 538 nm emission filters. ROS production was expressed as fluorescence intensity and expressed as a percentage of control cell.

Detection of apoptosis

Mitochondrial membrane potential, an early marker of apoptosis induction was assessed using MitolightTM Apoptosis Detection Kit (Chemicon International, Inc. Billerica, MA 01730, USA). Briefly, cells were cultured in the above reported experimental conditions and then incubated with the dye for 30 minutes at 37°C, as suggested by the kit protocol. The cells were placed on a microscope slide and observed immediately using a Zeiss Axiophot fluorescence microscope.

In healthy cells, the lipophilic cationic dye employed in the assay partitions to the cytoplasm and also accumulates in the form of red-fluorescent J-aggregates mitochondria, in a membrane potential-dependent fashion, due to the uptake by biochemically intact organelles. In apoptotic cells with altered mitochondrial membrane potential the dye is relocated to the cytosolic in the form of green-fluorescent monomers. Using filters to detect fluorescein and rhodamine, healthy cells are identified as containing red mitochondria against a green background of

cytoplasmic dye, while apoptotic cells appear nearly uniformally green.

In another set of experiments, the PC12 incubated with Mitolight in the conditions reported above, were immediately analyzed by Flow Cytometry (Coulter-Epics, MCL-XL). In this setting cell apoptosis was quantified by the number of cells detectable in the green fluorescence (FL-2) channel (a correlate for the leak of monomeric dye from depolarized mitochondria) vs. the number of cells detectable in the red fluorescence (FL-1) and expressed in percentage.

RNA isolation and semiquantitative PCR

Total RNA was isolated with SV Total RNA Isolation System (Promega Corporation 2800 Woods Hollow Road Madison, WI 53711, USA). RNA concentration was evaluated by spectrophotometric reading at 280 and 260 nm. Total RNA was used for first strand cDNA synthesis with HyperScript, First strand Synthesis Kit and Oligo-dT, as random primer (GeneAll). The PCR was performed with about 150 ng of cDNA using DreamTaq. The following primer sequences were used for amplification:

β-Actin forward 5′-CCTTCCTGGGCATGGAGT CCTG-3′,

β-Actin reverse 5′-GGAGCAATGATCTTGATCT TC-3′ (208 bp);

Bax forward 5′-GCAGGGAGGATGGCTGGGG AG-3′,

Bax reverse 5′-TCCAGACAAGCAGCCGCTCA CG-3′ (352 bp).

The experimental protocols for PCR reactions were initial denaturation for 5 minutes at 95°C; amplification for 40 cycles of denaturation, 30 seconds, 95°C, annealing: 30 seconds, at 55°C (β-Actin), 60°C (Bax) and elongation at 72°C for 1 minute; final elongation for 10 minutes at 72°C. PCR products were then analyzed by 1.5% agarose gels electrophoresis in TBE1X Buffer. Image acquisition and product analysis was made by Bio-Rad imaging systems with Quantity One1-D analysis software. The density of the PCR bands were divided by that of the housekeeping gene (β-Actin) and expressed as percent of the control band density.

Measurement of Caspase 3 activity

Caspase-3 activity was measured by using a specific assay kit from Sigma Chemical Co. (St Louis, MO, USA) following manufacture's instructions. DEVD-pNA was used as a colorimetric substrate. PC12 cells were plated at a density of 2.5×106 cells/35 mm dish and treated with 10 μM punicalagin 24 hours before the treatment with 300 micromolar H_2O_2. After 24 hours the cells were harvested by centrifugation. The pellets were washed with PBS, lysed in 50 ml of chilled cell lysis buffer and left on ice for 10 minutes. Lysate was centrifuged at $10\,000 \times g$ for 1 minute at 4°C, and supernatant was used for the Caspase-3 assay.

The protein concentration was confirmed by the BCA assay. The protease activity was determined by the spectrophotometric detection at 405 nm of the chromophore *p*-nitroanilide (pNA) after its cleavage by Caspase-3 from the labeled Caspase-3-specific substrate (DEVD-pNA). The Caspase 3 activity was expressed as percent of activity in the control cells.

Statistical analysis

Each experiment was repeated at least three times in single. All results were presented as the mean ± SEM of (*n*) replicates per experimental group. Data were subsequently analyzed by one-way ANOVA, followed by post hoc Newman–Keuls for comparisons between group means, or Dunnett test when appropriate using a PrismTM computer program (GraphPad, San Diego, CA, USA). Differences were considered statistically significant if $P < 0.05$.

Results

Effect of punicalagin on H_2O_2-induced cell viability and oxidation

In a first series of dose–response experiments, H_2O_2 was administered at dose-range 50–500 μM for 24 hours in order to determine the ideal cytotoxic dose to use in our experimental model. Figure 1A shows that cell viability declined in a dose-dependent manner, reaching the 50% of viability at concentration of 300 μM. Therefore, subsequent experiments were conducted used this concentration of H_2O_2 to cause cytotoxicity.

Afterwards, functional experiments were carried out to test the protective effect of punicalagin against H_2O_2-induced cytotoxicity in PC12 cells. Twenty-four hours pretreatment with punicalagin protected the cells from H_2O_2-induced toxicity. In fact the punicalagin, given in the range 0.5–20 μM, protected the cells in a concentration-dependent manner, recovering cell viability to about 85% at 10 μM (Figure 1B).

Cell viability data were also confirmed by the subsequent morphological studies conducted on cells in the same experimental paradigm (Figure 1C): the number of cells was markedly reduced and many of them had a rounded appearance after exposure to 300 μM H_2O_2 (Figure 1C/b), compared to control (Figure 1C/a); the pretreatment with 10 μM punicalagin resulted strongly protective (Figure 1C/c).

Further experiments were performed to ascertain whether protection effects caused by punicalagin were due to interference with ROS generation and to reduction of the oxidative stress. A significant and consistent increase in ROS was elicited by 300 μM H_2O_2 after 24 hours of treatment. Under these conditions,

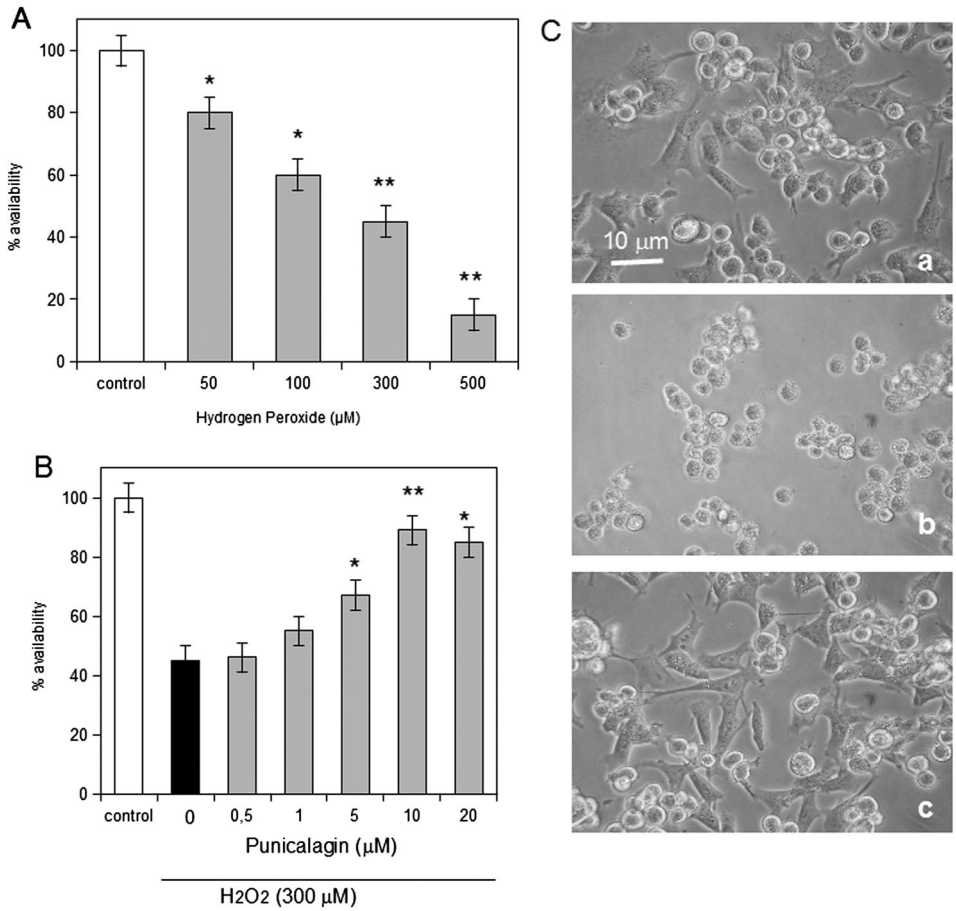

Figure 1 (A) Viability of PC12 cells after 24 hours treatment with H_2O_2 at different concentrations (50, 100, 300, and 500 μM). (B) Viability of PC12 cells pre-treated (24 hours) with punicalagin at different concentrations (0, 0.5, 1, 5, 10, and 20 μM) and subsequently co-treated with H_2O_2 (300 μM) for further 24 hours. The control represents the viability of PC12 cells untreated and the black bar the viability of PC12 cells treated with H_2O_2 without punicalagin. Cell viability was determined by MTS assay (see text). Data from six independent experiments are expressed as percent viability respect to cell untreated (control = 100%) and are represented by means ± SEM. In (A) * and **$P < 0.01$ and $P < 0.001$ vs. controls, respectively; in (B) * and **$P < 0.01$ and $P < 0.001$ vs. cells treated with H_2O_2 300 μM alone. (C) PC12 cell morphology observed by phase-contrast microscopy. (a) Control; (b) cells treated with H_2O_2 (300 μM) for 24 hours; (c) cells pre-treated for 24 hours with 10 μM punicalagin and successively with H_2O_2 (300 μM) for further 24 hours.

a 24 hours pre-treatment with punicalagin (range 0.5–20 μM) reduced the ROS production in dose-dependent manner, reaching value approximately similar to the control (Figure 2). Punicalagin was able to reduce the production of ROS stimulated by H_2O_2 in a significant manner from 10 μM onward (Figure 2). No effect of punicalagin alone on cell was observed both on cell viability and on ROS production (data not shown).

Punicalagin pretreatment protects PC12 cells against H_2O_2-reduced mitochondrial membrane potential

In view of the tight correlation between ROS production and apoptosis, we investigated the hypothesis of an anti-apoptotic effect by punicalagin after oxidative stress in PC12 cells. Apoptosis detection was performed using a MitolightTM Apoptosis Detection Kit. As evidenced by Figure 3A: control cells showed an intense red color (indicative of a healthy organelle) due to polymerization of dye in mitochondria, while cells treated with H_2O_2 showed an intense green fluorescence (indicative of apoptosis) due to an altered mitochondrial membrane potential. Interestingly, pre-treatment with 10 μM punicalagin for 24 hours significantly preserved the mitochondrial membrane potential up to a level comparable to untreated cells, indicating a protective effect able to oppose the H_2O_2-mediated apoptosis (Figure 3A).

In order to obtain a quantitative measure of cell death and protection by punicalagin, the PC12 cells were incubated with Mitolight in the conditions reported above and they were analyzed by flow cytometry (Figure 3B). In healthy cells, the dye accumulates and aggregates in the mitochondria, giving off a bright red fluorescence; while in apoptotic cells with altered mitochondrial membrane potential, the dye in its monomeric form stays in the cytoplasm, fluorescing green, providing a ready discrimination between apoptotic and non-apoptotic cells. The apoptotic

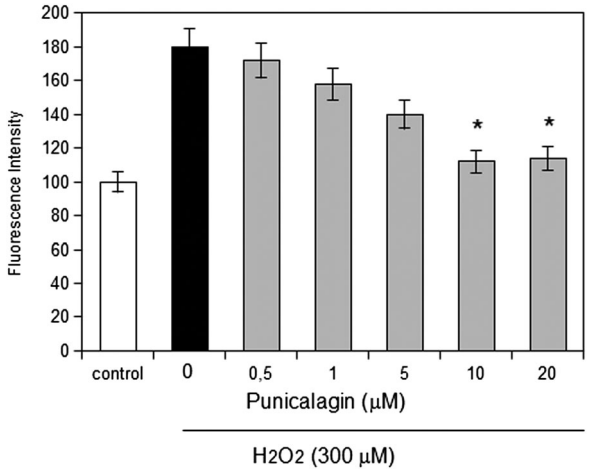

Figure 2 ROS production (expressed as fluorescence intensity) in PC12 cells pretreated with punicalagin at different concentration (0.5, 1, 5, 10, and 20 μM), 24 hours before of the treatment with H_2O_2 300 μM (24 hours). The control represents the viability of PC12 cells untreated and the black bar the viability of PC12 cells treated with H_2O_2 without punicalagin. Results are from six independent experiments and are expressed as percent viability respect to cell untreated (control = 100%). All values indicate means ± SEM. *$P < 0.01$ vs. cells treated with H_2O_2 300 μM alone.

cells were quantified by the number of cells detectable in the green fluorescence channel vs. the number of cells detectable in the red fluorescence channel and expressed in percentage. Three-hundred micrometer H_2O_2-induced apoptosis in 46% (±5) of cells, while pre-treatment with punicalagin induced apoptosis only in 6% (±5) of cells, value very similar to that obtained in the control (6% ± 1). No effect of punicalagin alone was observed (data not shown).

Effects of punicalagin on H_2O_2-induced Bax gene expression and Caspase-3 activation

To verify the involvement of mitochondria-mediated apoptotic pathways in PC12 cells treated with 300 μM H_2O_2 we investigated the Bax gene expression. As shown in Figure 4A, H_2O_2 exposure for 24 hours significantly up-regulated bax mRNA expression, whereas down regulation was observed in pretreated cell with punicalagin (10 μM for 24 hours). No differences were observed in bcl2 gene expression (data do not shown).

Caspase-3 has been reported to be a key performer of the Caspases involved in cellular apoptosis which modulates the mitochondria-dependent pathway.[14] Therefore it was of interest to determine whether Caspase-3 activation occurred after H_2O_2 exposure and whether punicalagin had a protective effect in the same experimental paradigm.

As shown in Figure 4B, treatment with H_2O_2 caused a remarkable increase of Caspase-3 activity. However, the addition of 10 mM punicalagin 24 hours before resulted in the reduction of Caspase-3 activity in a significant manner, with values very similar to control no H_2O_2-exposed. No effect of punicalagin alone was observed both on Bax gene expression and on Caspase-3 activity (data not shown).

Discussion

Present study shows that the pre-treatment with punicalagin increases neuronal resistance to H_2O_2-induced toxicity, significantly improving the cell viability of PC12 cells. The molecular mechanism underlying this observed phenomenon appears to be linked to a inhibition both of oxidative cellular potential and apoptotic signals (Bax and Caspase-3).

The findings highlighted in this study fit well with evidence of scientific literature showing that central neurons are vulnerable to insults induced by oxidative stress. In fact ROS, produced by mitochondrial damaged during oxidative stress,[15] can damage proteins, nucleic acids, and polyunsaturated fatty acids of cell membranes, which lose their integrity with increase of permeability to Ca^{2+}.[16] Moreover, in recent years, a number of studies have shown that oxidative stress could cause cellular apoptosis via both mitochondria-dependent and mitochondria-independent pathways.[17,18]

In this framework, one of the plausible ways to prevent neurological disorders induced by oxidative stress is to augment or potentiate the cellular defense capacity through a proper dietary regimen intake of antioxidants.[19] Scientific evidences show that pomegranate juice contains higher antioxidants levels than red wine, green tea, and other fruit juices, such as cranberry or blueberry.[20] For this reason in this study we have chosen the punicalagin (2,3-S-hexahydroxydiphenoyl-4,6-(S-S)-gallagyl-D-glucose), an ellagitannin isolated from pomegranate polyphenols, as potential natural protective molecule. In fact, it has been demonstrated that this molecule has a protective action against the oxidative stress-induced cell injury in many tissues,[21–23] by increasing endogenous catalytic antioxidant defense systems and reducing pro-apoptotic genes expression.[5,24] The molecular mechanisms underlying these observed phenomena appears to be linked to a control of oxidative cellular potential, because cells pre-treated with punicalagin maintain low levels of ROS compared to cells treated only with H_2O_2. In addition, a recent paper has showed also that punicalagin attenuates toxicity in hepatocytes by activating the nuclear erythroid 2-related factor 2 (Nrf2), a protein regulating the expression of antioxidants able to protect the cells against oxidative stress and apoptosis.[6] Interestingly, it has also been observed in PC12 cells that H_2O_2 activates MAP kinase, which mediate the production of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6)

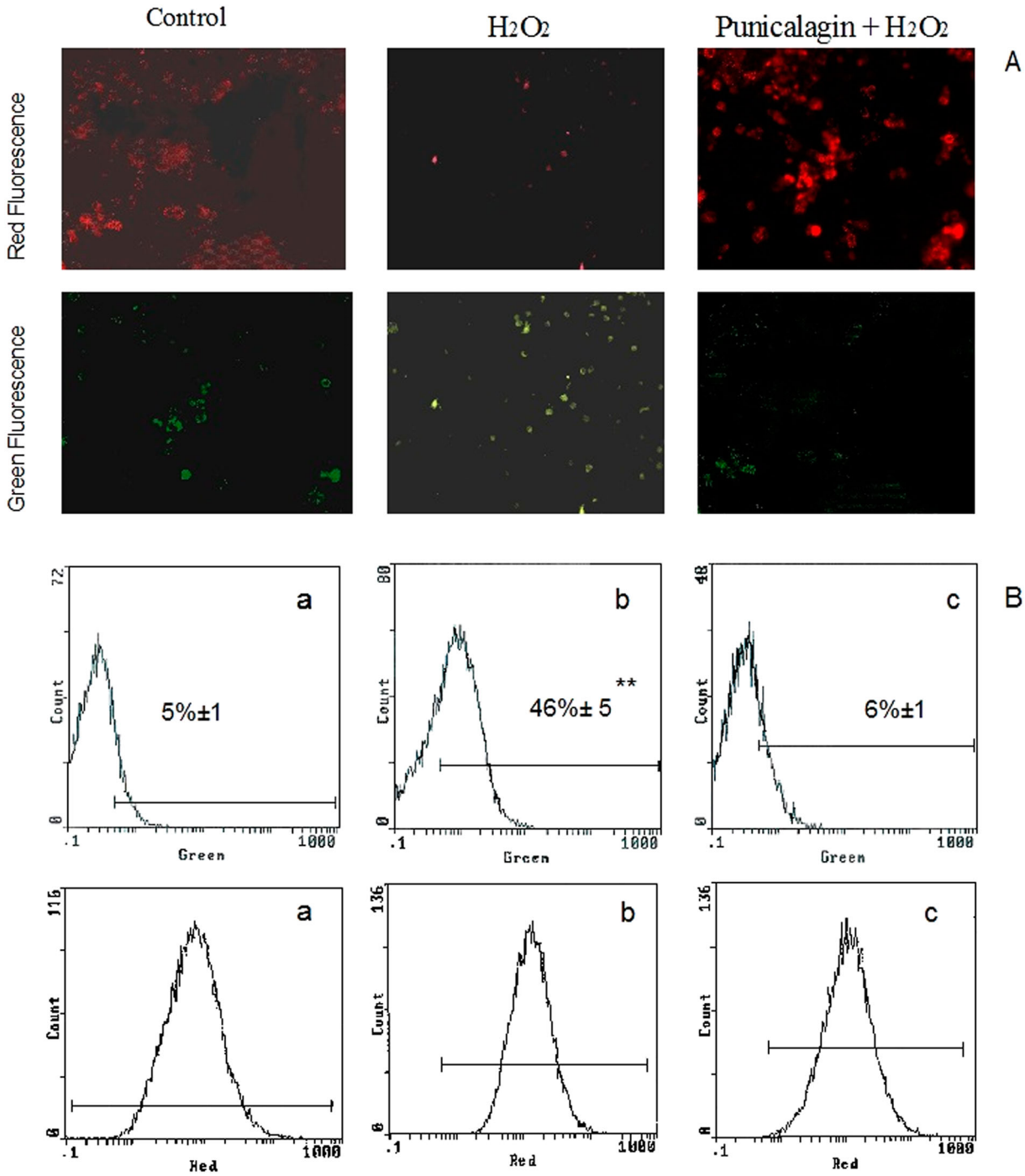

Figure 3 (A) Mitochondrial membrane depolarization evidenced with mitolight mitochondrial kit in spectrofluorometry in PC12 cell in different experimental conditions. Red fluorescence indicates the healthy cells (polarized mitochondria), green fluorescence indicates the presence of apoptotic cells (depolarized mitochondria). Data are representative of three independent experiments. (B) Quantitative analysis on PC12 cells. (a) Control; (b) cells treated with H_2O_2 300 μM for 24 hours; (c) cells pretreated for 24 hours with punicalagin 10 μM and successively with H_2O_2 300 μM for 24 hours. After treatments the cells were suspended in Mitolight solution and immediately analyzed by flow cytometer. Cell apoptosis was quantified by the number of cells detectable in the green fluorescence (top) channel vs. the number of cells detectable in the red fluorescence (bottom) and expressed in percentage. Data from three independent experiments are expressed as percent of apoptotic cells and are represented by means ± SEM. **$P < 0.001$ vs. control cells.

with consequent activation of the apoptosis through triggering of the Caspase cascade.[25]

Our study is a further support to the notion that punicalagin might reduce H_2O_2-induced cytotoxicity. Indeed, we found that Bax gene expression and Caspase-3 activity were activated by ROS production and that the pre-treatment with punicalagin prevents apoptotic phenomena, in PC12 cells. Furthermore we hypothesize that pre-treatment with punicalagin, preserves the cells by H_2O_2-induced mitochondrial membrane damage, increasing mitochondrial membrane potential. It is well known that the increased mitochondrial membrane

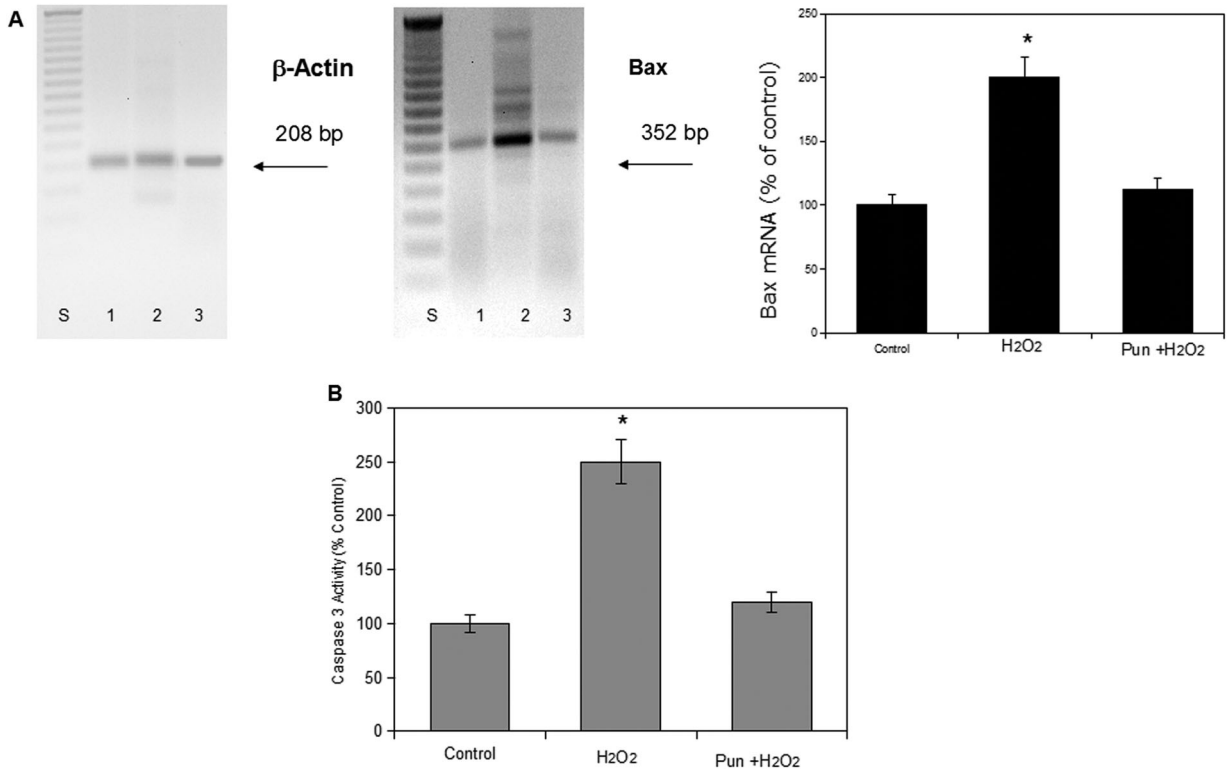

Figure 4 (A) Agarose gels representing mRNA expression levels for β-Actin (housekeeping gene) and for Bax in PC12 cells untreated (line 1), treated with H_2O_2 300 mM for 24 hours (line 2) and preconditioned with 10 μm punicalagin before of treatments with H_2O_2 (line 3). In the first line (S) is reported DNA ladder (50 pb). (A) (right): Bax densitometric analysis of the gels in figure to the side. The density of the gel bands was divided by that of the β-Actin and expressed as percent of the control band density. Results are from three independent experiments. Data are represented by means ± SEM. *$P < 0.01$ vs. Controls. (B) Caspase-3 activity (expressed as percent of control) in PC12 cells untreated; treated with H_2O_2 300 μM for 24 hours and pretreated for 24 hours with punicalagin 10 μM before of the treatment with H_2O_2 Results are from three independent experiments. Significantly different from controls *$P < 0.01$.

permeability is controlled by Bax, a pro-apoptotic factor, which increases the opening of the mitochondrial voltage-dependent anion channel that leads to loss of membrane potential and the release of cytochrome c.[26] So, when trans-located in the mitochondrial membrane, Bax can homodimerize and triggers the activation of terminal Caspases (in particular Caspase 3) through alteration of mitochondrial functions, which cause the release of factors that promote apoptosis into the cytoplasm.[27] Finally, Caspase-3, the main apoptotic executioner, causes chromatin condensation, protein breakdown, and DNA fragmentation.[28,29]

Hence the effects of punicalagin observed on apoptotic markers are surely due to a control of oxidative stress, but we cannot even rule out a direct control on apoptotic genes. In fact Chen *et al.*[24] in 2013, found that punicalagin attenuates directly hypoxia-induced apoptosis in syncytiotrophoblasts through (i) a reduced p53 activity, (ii) a decreased expression of p21, (iii) a lower HIF1α expression, and (iv) a limited activity of caspases 9 and 3. Moreover, it is well known that the antioxidant action of many bioactive molecules is not limited to ROS scavenging, and includes the modulation of cell signaling, gene expression and activity of antioxidant enzymes.[30]

In conclusion, this study shows for the first time the neuroprotective and anti-apoptotic effect of punicalagin on H_2O_2-induced cell death in PC12 cell. Furthermore, based on our observations, one can also imagine the punicalagin as a substance with a protective potential against neurodegenerative diseases. In fact, ROS are the trigger of the damage to the mitochondrial respiratory chain and of loss of mitochondrial membrane potential; all factors that mediate or amplify the neuronal dysfunction during the course of the neurodegeneration, and consequently implicated in the development of neurodegenerative diseases.[13,26] Note, in addition, that the protective effect exerted by punicalagin has been observed with a easily achievable concentration with a portion of fresh pomegranate juice where punicalagin levels reach the dose of 2g/l.[31]

Disclaimer statements

Contributors None.

Funding The present study was financed by the Italian National Research Council (CNR) funding.

Conflicts of interest None.

Ethics approval None.

Acknowledgements

The authors of the present study would like to thank Dr Lucia Lisi for her contribution to the experimental work described and for her expert technical assistance.

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