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Dose-dependent effect of Resveratrol on bladder cancer cells: Chemoprevention and oxidative stress

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ABSTRACT

Background: Over 6 million people die annually in the world because of cancer. Several groups are focused on studying cancer chemoprevention approaches. Resveratrol, a polyphenol, at high dosages, has been reported as antitumor and chemopreventive. However, it has a dose-dependent effect on cell death, even on some cancer cells.
Objectives: Our aim was to investigate this dose-dependent effect on human bladder carcinoma ECV304 cells during oxidative stress condition.
Methods: For this purpose, ECV304 cells incubated with different Resveratrol concentrations were analyzed as for their metabolic rate, membrane permeability, DNA fragmentation, anti/proapoptotic protein levels and phosphatidylserine exposure after oxidative stress.
Results: Resveratrol induced cell death at high concentrations (>20 μM), but not at low ones (0.1–20 μM). Pretreatment with 2.5 μM protected the cells from oxidative damage, whereas 50 μM intensified the cell death and significantly increased Bad/Bcl-2 ratio (proapoptotic/antiapoptotic proteins). Resveratrol was able to modulate NO and PGE_2 secretion and performed an anti-adhesion activity of neutrophils on PMA-activated ECV304 cells.
Conclusions: Resveratrol at high doses induces cell death of ECV304 cells whereas low doses induce protection. Modulation of Bcl-2 protein induced by Resveratrol could be mediating this effect. This information about the role of Resveratrol on cancer alerts us about its dose-dependent effects and could lead the design of future chemoprevention strategies.

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1. Introduction

Cancer is the largest single cause of death both in men and women, claiming over 6 million lives each year worldwide. Chemoprevention, the prevention of cancer by ingestion of chemical agents that reduces the risk of carcinogenesis [1], is one of the most direct ways to reduce morbidity and mortality. Public health policy initiatives have, thus, been focused on the "chemoprevention" of cancer, wherein occurring naturally or when synthetic chemical agents are used for the inhibition, delay or, even, reversal of carcinogenesis [2].

Resveratrol, a polyphenol derived from red grapes, berries, and peanuts, has been shown to modulate death of a wide variety of cells and also has been linked with suppression of inflammation, arthritis, cardiovascular diseases, and delaying of aging [3]. Resveratrol is an excellent scavenger of hydroxyl, superoxide, and other radicals [4]. It also protects against lipid peroxidation in cell membranes and DNA damage caused by reactive oxygen species generation [4]. Resveratrol was further demonstrated to be an antitumor and chemopreventive agent; and found to affect cellular proliferation through its action on tumor initiation, promotion, and progression [5,6]. These properties have been explained mainly by its activities in cell cycle control and apoptosis induction [7,8].

A preliminary study has investigated the chemopreventive potential of Resveratrol against bladder cancer (T24 cells line) and its mechanism of action [9]. It has been demonstrated that treatment of bladder cancer cells with high doses of Resveratrol (>25 μM) resulted in a significant decrease in cell viability by inducing apoptosis and cell cycle arrest. In fact, Resveratrol has been reported to facilitate apoptotic cell death and it behaves as a chemopreventive alternative when administered in higher doses [10]. On the other hand, at very low concentrations (<20 μM), this same Resveratrol is able to inhibit apoptotic cell death, providing, thereby, protection against various diseases, including myocardial ischemic reperfusion injury, atherosclerosis, and ventricular

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arrhythmias [3]. Thus, Resveratrol plays a dose-dependent biphasic effect on death cell, even on cancer cells [11]. The present paper was designed to study the dose-dependent effect of Resveratrol on cell viability and its antioxidant role on ECV304 cells, a derivation from the human bladder carcinoma T24 cell line. Moreover, the Resveratrol activity as an inflammatory modulator molecule was also evaluated on those cells through nitric oxide (NO) and prostaglandin E_2 (PGE_2) analysis.

2. Methods

2.1. Preparation of Resveratrol solutions

Resveratrol was purchased from Sigma–Aldrich (St. Louis, USA; catalog number: R5010), dissolved in DMSO (dimethyl sulfoxide; Sigma–Aldrich), and stored at −20 °C. For all experiments, the final concentrations of Resveratrol were prepared by diluting the stock in RPMI-1640 medium and then, in sterilized filter. The final concentration of DMSO in cultured medium was less than 0.5%, which caused no measurable effect on cell growth.

2.2. Treatment of cell culture

In all experiments were used bladder carcinoma cell line ECV304, a derivation from the human bladder carcinoma T24 cell line (American Type Culture Collection, Manassas, USA). This line was cultivated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, USA), 2 mM L-glutamine, 0.075% sodium bicarbonate, 100 U/ml penicillin and 10 mg/ml streptomycin (Invitrogen, Life Technologies, Carlsbad, USA) at 37 °C in 5% CO_2. Confluent ECV304 cells were previously incubated with different Resveratrol concentrations (0.1–100 μM). After 30 min at 37 °C, oxidative stress was induced by adding 50 μM H_2O_2 (hydrogen peroxide) which was maintained for 6 h [12]. In this protocol, total time of Resveratrol exposition was 6 h 30 min. At the end of the incubation period, different parameters were analyzed as described below. When necessary, normal fibroblast cells (LLCMK2, ATCC CCL-7.1) were incubated with Resveratrol. This cell line was cultivated in the same conditions that ECV304 cells.

2.3. MTT assay

The cell metabolism of ECV304 cells was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described [13]. Briefly, confluent ECV304 cells cultivated into 96-well culture plates were treated with various concentrations of Resveratrol for 6 h 30 min. Triton (10%) was used as death control. After incubation, the medium was removed, the cells were washed with phosphate-buffered saline (PBS) and, subsequently, incubated with 0.5 mg/ml MTT (Sigma–Aldrich) for additional 4 h. Later, 0.2 ml of lysis buffer (isopropyl alcohol containing 10% Triton X-100 and 0.1 N HCl) was added to dissolve the formazan crystals, and then, the absorbance was measured in a spectrophotometer at 570 nm.

2.4. Flow cytometric analysis

Cells (10^5) cultivated in 12-well culture plates were appropriated treated with RSV and stimulated or not with H_2O_2. Apoptosis and necrosis signals were investigated in these cells by propidium iodide (PI; Sigma–Aldrich) staining. Membrane permeability in fresh ECV304 cells was evaluated immediately after PI addition (5 μg/ml). DNA degradation was detected in ECV304 cells gently resuspended in 0.5 ml of hypotonic propidium iodide solution (PI, 50 μg/ml in 0.1% sodium citrate plus 0.1% Triton X-100). The tubes were kept at 4 °C for 16 h in the dark.

In order to investigate the phosphatidylserine exposition on ECV304 cell surface, at the end of incubation these cells with RSV and/or H_2O_2 they were washed at least twice in sterile PBS and, then, incubated in a 100-μl final volume in HEPES buffer (10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4) containing Alexa 488-conjugated AnnexinV (2:100) (Invitrogen, Life Technologies, Eugene, USA) for 15 min at 4 °C. After incubation it was added a buffer and propidium iodide (PI). Samples were acquired immediately by flow cytometry.

Nitric oxide intracellularly generated was measured by using 4,5-diaminofluorescein diacetate (DAF-2 DA; Sigma–Aldrich), which is membrane permeable [14]. ECV304 cells (10^6) were incubated with different Resveratrol concentrations (0–50 μM) for 60 min in the presence of DAF-2DA (2 μM). Cells were washed twice in PBS and immediately acquired by flow cytometry.

In all assays the samples were acquired by flow cytometry FACS Canto (Becton Dickinson, Mountain View, USA) and analyzed in the DIVA software (Becton Dickinson).

2.5. DNA fragmentation

Cells were treated as previously described, centrifuged, resuspended in 500 μl of lysis buffer (Tris 10 mM, EDTA 1 mM, 0.2% Triton X-100, pH 7.4), and incubated overnight at 4 °C. The samples were centrifuged and the supernatants were treated with 100 μg/ml RNAse (Invitrogen) for 1 h at 37 °C followed by 0.1 mg/ml proteinase K (Invitrogen) for 1 h at 57 °C (134.6 °F). Nucleic acids were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) (BioAgency, São Paulo, BR) and precipitated with ethanol (P.A) [15]. After ethanol precipitation, nucleic acid was resuspended in TE buffer (10 mM Tris–HCl pH 7.4, 10 mM EDTA). Finally, the resuspended DNA was subjected to electrophoresis in a 2.0% agarose gel at a constant voltage of 40 V for 2 h [16].

2.6. Immunoblotting

After treatment with Resveratrol, cultures were washed twice with PBS and lyzed in boiling sample buffer (62.5 M Tris, pH 6.8, 2% SDS (w/v), 5% glycerol (v/v), 30 μM phenol red and 0.9% β-mercaptoethanol). Samples were incubated for 5 min at 100 °C, centrifuged, and loaded in 15% polyacrylamide gels. Nitrocellulose electroblotted membranes (Amersham Biosciences, Uppsala, Sweden), saturated with 5% non-fat dry milk, were sequentially incubated with primary and secondary antibodies, washed in TBS-T and revealed using a super-signal luminol substrate (Pierce). Densitometry analyses were done using ImageJ software. Antibodies for Bcl-2 (1:1000; catalog number 551109), Bad (1:500; catalog number 610391) and caspase-3 (1:1000; catalog number 557035) were obtained from BD Pharmingen. Antibodies for caspase-9 (1:250; catalog number C4356) and tubulin (1:1000; catalog number T3320) were obtained from Sigma. Secondary antibodies were obtained from Jackson ImmunoResearch Inc.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The levels of PGE_2 were determined in the supernatant of cultures previously treated with Resveratrol (0–50 μM) for 24 h at 37 °C. A competitive immunoassay was performed following manufacturer's instructions (PGE_2 assay kit R&D Systems, Minneapolis, USA). The colorimetric reaction was measured in spectrophotometer at 450 nm. The detection limit of these assays was 10 pg/ml. Lipopolysaccharide (LPS, 1 μg/ml) was used as positive control to PGE_2 production.

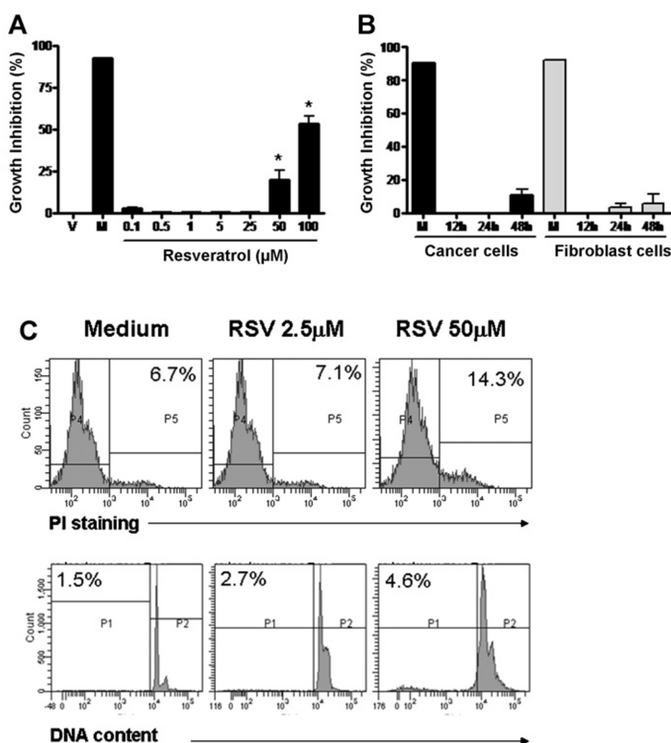

Fig. 1. Resveratrol presents a dose-dependent effect on ECV304 cell viability. ECV304 cells were incubated with Resveratrol (0.1–100 μM) during 6 h 30 min. In these conditions were analyzed the (A) cell viability by MTT assay and (C) the permeability membrane (PI staining), as well as the DNA content by flow cytometry. (B) Bladder cancer cells (ECV304) and fibroblast cells (LLCMK2) were incubated with 2.5 μM Resveratrol during different times and analyzed by MTT assay. In (A) and (B) data are reported as mean from three independent experiments ± S.E.M. $^*p < 0.05$ compared to viable cells (V). In (C) results shown are representative of three independent experiments done in triplicate. V, cells incubated with medium alone; M, cells incubated with 10% Triton-X; P4, not permeable plasmatic membrane; P5, permeable plasmatic membrane; P1, fragmented DNA; P2, not fragmented DNA.

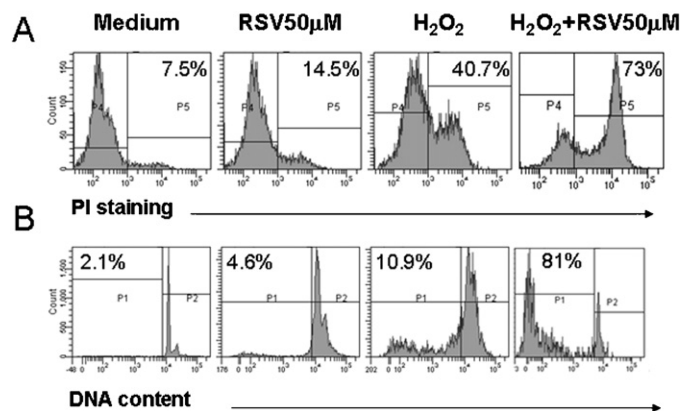

Fig. 2. Resveratrol at high dose increases the oxidative stress cell damage on ECV304 cells. The cells were previously incubated with Resveratrol (50 μM) for 30 min. H_2O_2 (50 μM) was added and maintained for 6 h in the culture. Permeability membrane (PI staining) and DNA content were analyzed by flow cytometry. The results shown are representative of three independent experiments done in triplicate. P4, not permeable plasmatic membrane; P5, permeable plasmatic membrane; P1, fragmented DNA; P2, not fragmented DNA.

2.8. Adhesion assay

Adhesion assays were performed in 24-well culture plate containing inside sterile cover slips. Human neutrophils were isolated as described [17] and added to monolayers of confluent ECV304 cells previously stimulated with PMA (Phorbol Myristate Acetate; 10 ng/ml, Sigma–Aldrich) for 5 h in the presence of 5 μM Resveratrol. At the end of incubation time, the adherent neutrophils were stained using HEMA3 (Biochemical Sciences, Inc., Swedesboro, USA), counted and expressed as number of neutrophils/field.

2.9. Statistical analysis

Statistical comparison was carried out with three or more groups using one-way analysis of variance (ANOVA) and Tukey's test. The data represent mean ± S.E.M. and values of $p < 0.05$ were statistically significant.

3. Results

3.1. Effect of Resveratrol on ECV304 cell viability

To evaluate if Resveratrol has any effect on ECV304 cell viability, these cells were treated with different concentrations of Resveratrol (0.1–100 μM) for 6 h 30 min at 37 °C. First, cell viability was measured by MTT assay and showed as growth inhibition (%) (Fig. 1A). Cells incubated with medium alone presented 100% of viability. Low concentrations of Resveratrol (0.1–25 μM) were not able to inhibit cell growth of ECV304. These results were maintained even when 2.5 μM Resveratrol was tested in additional times (Fig. 1B). In addition, the same Resveratrol concentration was not able to inhibit the growth cellular of fibroblast cells (Fig. 1B). However, ECV304 cells incubated with higher Resveratrol concentrations (50–100 μM) have shown statistically significant decrease in their cell growth (Fig. 1A). Using a second experimental approach, the cells were incubated with low (2.5 μM) and high (50 μM) doses of Resveratrol during 6 h 30 min. By flow cytometry, the cell permeability (PI staining) and DNA content were analyzed (Fig. 1C). The treatment of cells with Resveratrol at low doses did not induce statistically significant changes when compared to the cells that received only medium. On the other hand, a high dose of Resveratrol succeeded in increasing the cell permeability (14.3%) and the loss of DNA content (4.6%) (Fig. 1B).

3.2. Dual effect of Resveratrol, anti and pro-oxidant, on bladder carcinoma cell line ECV304

Oxidative stress induced by 50 μM H_2O_2 was used to test the anti and pro-oxidative activities of Resveratrol on bladder carcinoma cell line ECV304. For this purpose, different parameters, such as cell permeability, DNA content and phosphatidylserine exposure, were assayed. ECV304 cells were pretreated with 50 μM Resveratrol for 30 min and then 50 μM H_2O_2 was added to the cell culture. After 6 h of incubation, the signals of cellular damage induced by the oxidative stress were analyzed by flow cytometry assays (Fig. 2). Cells incubated with medium alone showed a basal level of cell permeability (7.5%), which was increased to 40.7% when H_2O_2 was added to the culture. The percentage of cells positive to PI staining (necrotic cells) was highlighted in the presence of Resveratrol at high dose (73%) (Fig. 2). In the same way, the DNA damage induced by oxidative stress (10.9%) has increased (81%) by 50 μM of Resveratrol (Fig. 2). Untreated cells presented 2.1% of DNA lost (apoptotic signal).

In parallel, ECV304 cells maintained in oxidative stress conditions were pretreated with 2.5 μM Resveratrol for 30 min as described above. ECV304 cells incubated with medium alone showed basal levels of cell permeability (9.1%) (Fig. 3A) and loss of DNA content (5.6%) (Fig. 3B). Incubation with 2.5 μM Resveratrol alone did not induce statistically significant cell modifications. On the other hand, when 50 μM of H_2O_2 was added to the cell culture, around 47% of the cells lost plasma membrane integrity and

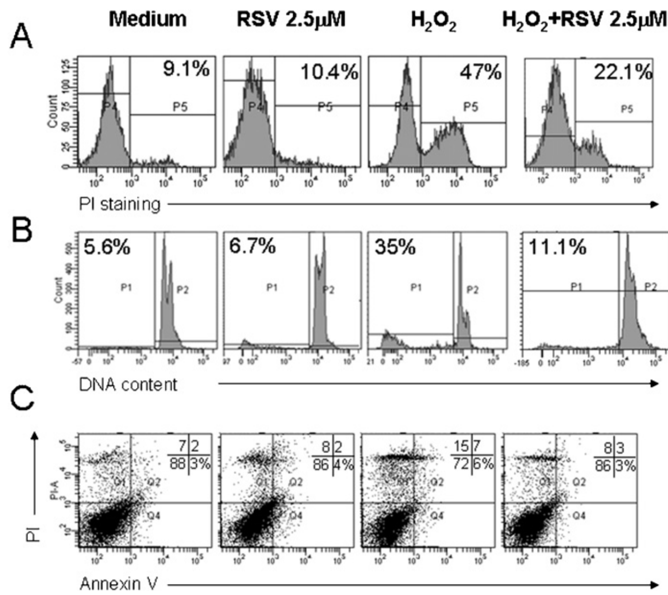

Fig. 3. Resveratrol at low dose decreases the oxidative stress cell damage on ECV304 cells. Cells were previously treated with 2.5 μM Resveratrol for 30 min and then 50 μM H_2O_2 was added to the cell culture. After 6 h of incubation the cells were analyzed by flow cytometry: (A) permeability membrane by PI staining; (B) DNA content; and, (C) double-stained using AnnexinV and PI. The results shown are representative of three independent experiments done in triplicate. P4, not permeable plasmatic membrane; P5, permeable plasmatic membrane; P1, fragmented DNA; P2, not fragmented DNA; Q1, necrotic; Q2, late apoptotic; Q3, living; Q4, early apoptotic cells.

around 35% of them presented loss of DNA content (Fig. 3A and B). This way, the presence of RSV during the oxidative stress induced by H_2O_2 significantly reduced the cell damage signals, by 22.1% of cell permeability and approximately 11.1% lost DNA content.

The phosphatidylserine was evaluated in association with the cell permeability for a better evaluation and characterization of cell death (Fig. 3C). We considered $PI^-AnnexinV^-$ cells as viable ones (Q3 region), $PI^+AnnexinV^-$ cells as necrotic ones (Q1 region), $PI^-AnnexinV^+$ as apoptotic ones (Q4 region) and $PI^+AnnexinV^+$ as cells in later apoptosis process (Q2 region). Thus, we could observe that ECV304 cells incubated with medium or Resveratrol alone presented similar percentage of apoptotic, necrotic and late apoptotic cells, 3%, 7% and 2% respectively. Oxidative stress enhanced percentages of necrotic cells (15%) ones and later apoptotic cells (7%). The pre-incubation of the cells with RSV reduced the damaged cells to similar levels as those incubated with medium alone (Fig. 3C).

Finally, we regarded the presence of DNA degradation in the cells pretreated with RSV and incubated with H_2O_2 (Fig. 4). DNA samples from ECV304 cells cultivated in different conditions were assayed in agarose gel by GelRed staining. The treatment of cells with H_2O_2 induced a strong DNA degradation when compared to samples from cells incubated with medium or RSV alone. However, the extension of DNA degradation induced by H_2O_2 was reduced when the cells were previously treated with RSV for 30 min.

3.3. Resveratrol treatment modulates levels of Bcl-2 and Bad proteins

Studies have suggested that Resveratrol alters cell viability by modulating Bcl-2 protein families, which are involved in promoting the apoptosis process [18,19]. We examined the effect of Resveratrol treatment on two members of Bcl-2 protein family (Bcl-2 and Bad) in ECV304 cells. As shown by immunoblot analysis and its densitometric quantification (Fig. 5), Resveratrol treatment of ECV304 cells at high doses (RSV 50) resulted in a decrease in antiapoptotic Bcl-2 and a concomitant increase in proapoptotic Bad proteins. This

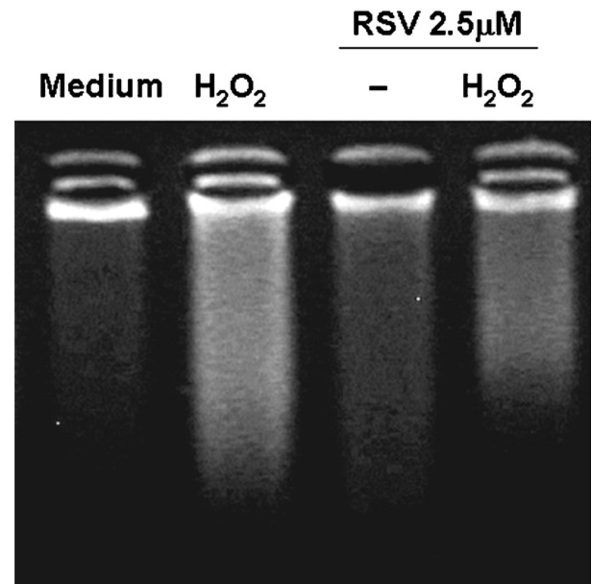

Fig. 4. Resveratrol protects ECV304 cells against DNA degradation induced by oxidative stress. Cells were previously treated with 2.5 μM Resveratrol for 30 min and then 50 μM H_2O_2 was added to cell culture. After 6 h of incubation the cells were processed as described in Section 2. DNA fragmentation was analyzed by electrophoresis in agarose gel. The result shown is representative of three independent experiments done in triplicate.

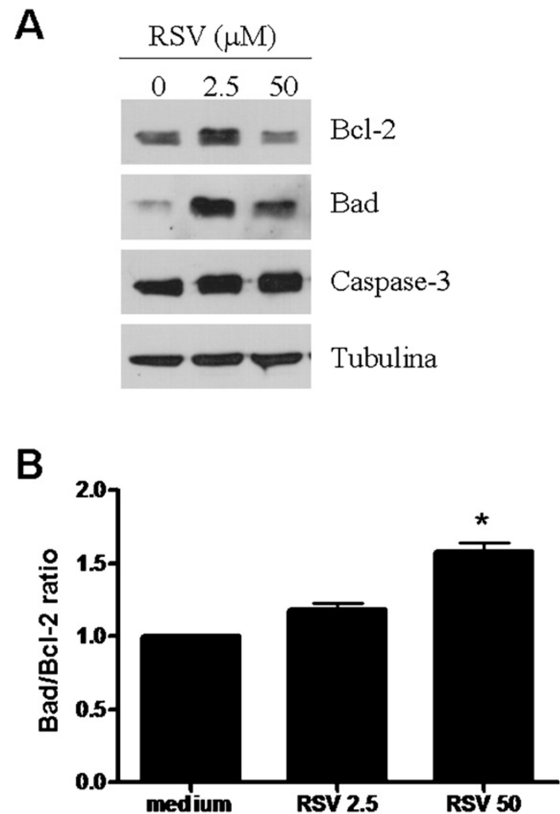

Fig. 5. Effect of Resveratrol on the levels of proteins involved in cell death control. ECV304 cells were incubated with Resveratrol (2.5 or 50 μM) during 6 h 30 min and analyzed by immunoblotting for specific proteins. Equal loading was confirmed by stripping the blot and reprobing it for tubulin (endogenous control). (A) Bands are from a representative experiment repeated three times with similar results. (B) Bad/Bcl-2 ratio from relative density normalized to tubulin. Data are reported as mean ± S.E.M. from three independent experiments done in triplicate. $^*p < 0.05$ compared to medium alone.

effect caused a significant increase in the Bad/Bcl-2 ratio (~51%) that favors apoptosis. On the other hand, Resveratrol treatment at low doses increased both proteins, Bcl-2 and Bad. In this condition, there was a smaller increase of Bad/Bcl-2 ratio (~29%) compared to that at high doses. The levels of caspase-3 during Resveratrol treatment were also assessed. In any condition, Resveratrol treatment was able to alter the caspase-3 levels (Fig. 5).

3.4. NO and PGE_2 generation in Resveratrol-treated cells

We tested the effects of the Resveratrol on NO and PGE_2 synthesis (Fig. 6). We administered to ECV304 cells increasing concentrations of Resveratrol (0–50 μM) and the NO production was measured using the NO sensitive fluorescent dye DAF-2 DA. In order to determine whether NO is produced or not produced as a result of treating ECV304 cells with Resveratrol, the fluorescence signal on cells loaded with DAF-2 DA was measured before and after the addition of Resveratrol. After 60 min of treatment we could observe that all Resveratrol concentrations tested were able to induce NO release compared with the control (cells incubated with medium alone) (Fig. 6A). In contrast, the presence of PGE_2 was not detected by immunoenzymatic assay on the supernatant from ECV304 incubated with different Resveratrol concentrations (Fig. 6B). Whereas, our positive control, LPS, has succeeded in stimulating the ECV304 cells to secrete more than 150 pg/ml of PGE_2.

3.5. Resveratrol effect on neutrophil adhesion on ECV304 cells

Neutrophil adhesion on ECV304 cells, an important event during inflammatory process, was assayed in the presence of Resveratrol. For this purpose, human purified neutrophils were added on ECV304 cells monolayer previously incubated with PMA in the presence of Resveratrol (Fig. 6C). Adherent neutrophils were counted and expressed as neutrophils/field. ECV304 cells not stimulated with PMA and cultivated with medium or Resveratrol alone presented a basal number of adherent neutrophils (Fig. 6C). This number was increased when the ECV304 cells were stimulated with PMA in the absence of Resveratrol. On the other hand, the presence of Resveratrol has inhibited 50% of the neutrophil adhesion induced by PMA.

4. Discussion

Resveratrol is currently being evaluated as a potential cancer chemoprevention agent against several kinds of tumors such as leukemia, prostate, breast, and colon cancers [19–21]. Previous work has demonstrated that a high dose of Resveratrol is able to induce apoptosis and cell cycle arrest of human T24 bladder [9]. However, there are a lot of reports describing the dual effects of Resveratrol on cell viability on dose-dependent manner, including normal and cancer cells [3,10,19,20,22]. Thus, the aim of the present study was to evaluate the effects of Resveratrol, at low and high doses, on cell viability of bladder carcinoma cell line ECV304 during an oxidative stress condition.

Briefly, we have observed a biphasic effect of Resveratrol on cell viability of ECV304 cells, since at high concentrations (>20 μM) it has induced death cell, whereas at low concentrations (0.1–20 μM) it has not and also protected them from oxidative stress. In addition, Resveratrol at low doses modulated the secretion of NO and PGE_2 by ECV304 cells and played an anti-adhesion activity of neutrophils on PMA-activated cells.

Cell viability of bladder carcinoma cell line ECV304 was evaluated by MTT assay and flow cytometry in the presence of different Resveratrol concentrations (0.1–100 μM). ECV304 cells present some advantages for this study: (i) they are a derivation from the

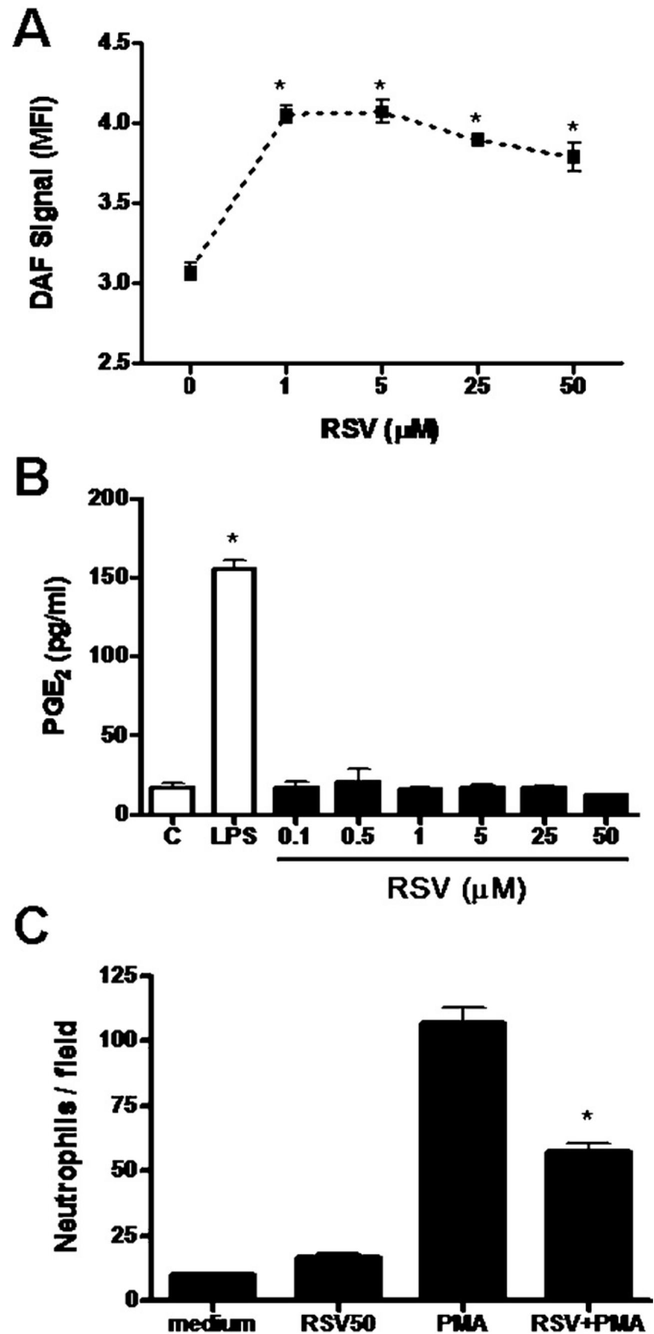

Fig. 6. Resveratrol modulates NO and PGE_2 synthesis and plays anti-inflammatory role. (A) ECV304 cells were pretreated with 2 μM of DAF-2 A and subsequently Resveratrol (0–50 μM) was added in the culture. After 1 h, intracellular NO production was analyzed by flow cytometry. (B) Resveratrol (0–50 μM) was maintained for 24 h in the culture. PGE_2 was quantified by immunoenzymatic assay. Medium alone (C) and LPS (1 μg/ml) were used as negative and positive controls, respectively. (C) Cells were stimulated with 10 ng/ml of PMA (Phorbol Myristate Acetate) for 5 h in the presence or absence of 5 μM Resveratrol. It was allowed neutrophil adhesion for 45 minutes. The adherent neutrophils were counted and expressed as number of neutrophils/field. All data are reported as mean ± S.E.M. from three independent experiments done in triplicate. $^*p < 0.05$ compared to RSV 0 μM in (A), to medium alone or C in (B) and to PMA in (C).

human bladder carcinoma T24 cell line, (ii) produce some inflammatory mediators, (iii) appropriated for metabolism studies and (iv) are easily maintained *in vitro* culture [23]. Our data show that, at low concentrations (0.1–25 μM), Resveratrol decreases neither cell viability nor DNA content, and does not induce a significantly cell permeability. On the other hand, at highest concentrations tested

(>25 μM), it was able to decrease the cell growth such as the DNA content and to increase the percentage of cells that present permeability, *i.e.*, signals of death cell. This biphasic effect of Resveratrol already has been demonstrated to other kind of cells, including normal and cancer cells [11,24]. In prostate cancer cells, the effect of Resveratrol on DNA synthesis varied dramatically, depending on the concentration and the duration of treatment. Using an extended treatment it was observed a dual effect of Resveratrol on DNA synthesis. At 5–10 μM it caused a 2- to 3-fold increase in DNA synthesis, and at >15 μM, it inhibited DNA synthesis [11]. A second study reported that low doses (0.1–1.0 μg/ml) of Resveratrol enhance cell proliferation, higher doses (10.0–100.0 μg/ml) induce apoptosis and decrease mitotic activity in colon cancer cells and endothelial cells [24]. Together, our results clearly corroborate with other previous ones about the importance of concentration to diverse activities of Resveratrol. Moreover, we have added new information about the effects of Resveratrol on bladder cancer cells since this polyphenol can induce or not these cells to die, depending on the dose used in a particular situation.

In an effort to try to understand better the effects of low concentrations of Resveratrol on bladder carcinoma cell line ECV304 we have investigated its antioxidant role on these cells. Resveratrol at low concentration (2.5 μM) was suited to play a protective effect on ECV304 cells from damage induced by oxidative stress. These conclusions were constructed from PI staining, DNA content, phophatidylserine exposure and DNA fragmentation assays. Interestingly, Resveratrol has induced release of nitric oxide from ECV304 cells. Reactive oxygen species (ROS) have been regarded as toxic products of metabolism because of their damaging effects on structural and functional molecules that, eventually, result in apoptosis and uncontrolled proliferation [25]. Although, Resveratrol has induced secretion of ROS from ECV304, it protected them from exogenous oxidative stress such as other natural antioxidants from vegetable foods that can counteract the effects of ROS [22]. Our group has demonstrated previously that soy extracts are also apt to activate NO synthesis in ECV304 cells and to protect them against cell damage [26]. According to the same authors, this antioxidant activity played by Resveratrol can be traced back to its cancer chemoprevention ones [5,27], since free-radical-mediated oxidative damage of DNA would be involved with the development of cancer cells [28]. On the other hand, more and more experimental evidence suggests that natural antioxidants play a chemopreventive role in an independent-manner on their ability to scavenge reactive oxygen species [2]. In face of these bases, we think that more investigations are necessary to elucidate the molecular events contributing to the antioxidative effect of Resveratrol on cancer cells.

The molecular mechanisms underlying the dual effect of Resveratrol on cell viability are not well understood. Some studies have shown that Resveratrol is able to modulate proteins involved with the control of apoptosis in mammalian cells [18,19]. Bcl-2 protein families are important regulators of death cell. Our results demonstrate that the treatment with Resveratrol alters the expression levels of Bcl-2 protein families on a dose-dependent manner. In summary, at low or high doses, Resveratrol increases the Bad levels, a proapoptotic protein. On the other hand, the levels of Bcl-2, an antiapoptotic protein, are significantly reduced when the cells are treated with high doses of Resveratrol. The densitometric quantification analysis indicates that Resveratrol down-modulates the Bad/Bcl-2 ratio by 29% at low doses and 51% at high doses. Although Resveratrol, at low or high doses, induces the increase of proapoptotic protein levels (Bad), it significantly decreases the expression of Bcl-2, an antiapoptotic molecule, when assayed at high doses. It is believed that, the different susceptibility to cell death shown by ECV204 from natural or oxidative stress is mediated by the modulation of Bcl-2 expression induced by different doses of Resveratrol. In addition, the death of ECV304 cells induced by high doses of Resveratrol is a caspase-independent process since caspase-3 levels were not altered.

Finally, we have evaluated some aspects related to the regulation of inflammation process by Resveratrol on ECV304 cells, since chronic inflammation can lead to cancer and other numerous diseases (diabetes, and cardiovascular, pulmonary, and neurological diseases) [3]. Resveratrol did not stimulate PGE_2 secretion, but it was efficient to inhibit the neutrophil adhesion. These results appoint Resveratrol as an important modulator of pivotal points during an inflammatory response, *i.e.*, secretion of proinflammatory molecules and inflammatory cell adhesion. Moreover, we reinforce the idea that Resveratrol is a nonsteroidal anti-inflammatory molecule and possess potential application in the treatment and prevention of various inflammatory diseases.

The consumption of polyphenols used as hormone therapy, mainly by postmenopausal women, has been widely accepted. Resveratrol is abundant in red wine with concentrations ranging from 10 to 20 μM [6,7]. The works show that Resveratrol is rapidly absorbed and metabolized. After consumption of wine, the concentration of free form of Resveratrol and its metabolites in human plasma is great inter-individual variable. The maximum plasma concentration was observed 1 h after oral intake and a second peak was present after 6 h [29]. These data, in association with our results, suggest that moderate consumption of red wine (2–3 glasses/day) can proportionate a low dose of serum Resveratrol by enough time to protect the cells from oxidative damage but cannot kill cancer cells.

Further studies are required to better characterize its dual effects on cell death, mainly on cancer cells. Our findings clearly demonstrate that, at low concentrations, Resveratrol does not kill the bladder carcinoma cell line ECV304. In contrast, it protects them from oxidative stress damage and stimulates an anti-inflammatory environment. On the other hand, high doses of Resveratrol induce cell death and increases cell damage from oxidative stress. Our data suggest that these effects are mediated by Bcl-2 antiapoptotic protein, which is down-modulated by high doses of Resveratrol. Several chronic medical conditions can develop after menopause: cardiovascular disease, osteoporosis, weight gain, urinary incontinence and some types of cancers, such as bladder cancer. Studies have been demonstrated that postmenopausal women present a statistically significant risk of bladder cancer compared with premenopausal women [30]. During our text, we described that Resveratrol is used as alternative hormone replacement therapy for these women. More recently its importance as antitumoral agent was reported. We believe that our results provide useful information for antioxidant and chemoprevention drug design. In addition, they can assist postmenopausal women to better choice their hormone therapy jointly their doctors.

Contributors

Bianca Stocco was responsible for writing the manuscript, the experiments: MTT assay, flow cytometric, analysis DNA fragmentation and help in immunoblotting and the statistical analysis of these experiments. Karina Toledo was responsible for writing the manuscript, immunoblotting and the adhesion assay and statistical analysis of these experiments. Mirian Salvador did MTT, enzyme-linked immunosorbent assay (ELISA) and statistical analysis of these experiments. Michele Paulo did flow cytometric to analyze nitric oxide. Natália Koyama did adhesion and MTT assay and Maria Regina Torqueti is the Project Coordinator.

Competing interests

The authors declare that they have no competing interests.

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