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GYNECOLOGIC ONCOLOGY

Vitamin K3 induces antiproliferative effect in cervical epithelial cells transformed by HPV 16 (SiHa cells) through the increase in reactive oxygen species production

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Abstract

Purpose Cervical cancer is characterized as an important public health problem. According to latest estimates, cancer of the cervix is the fourth most common cancer among women. Due to its high prevalence, the search for new and efficient drugs to treat this infection is continuous. The progression of HPV-associated cervical cancer involves the expression of two viral proteins, E6 and E7, which are rapidly degraded by the ubiquitin–proteasome system through the increase in reactive oxygen species generation. Vitamins are essential to human substances, participate in the regulation of metabolism, and facilitate the process of energy transfer.

Methods Some early studies have indicated that vitamin K3 exerts antitumor activity by inducing cell death by apoptosis through an increase in the generation of reactive oxygen species. Thus, we evaluated the antiproliferative effect and a likely mechanism of action of vitamin K3 against cervical epithelial cells transformed by HPV 16 (SiHa cells) assessing the production of total ROS, the mitochondrial membrane potential, the cell morphology, the cell volume, and the cell membrane integrity.

Results Our results show that vitamin K3 induces an increase in ROS production in SiHa cells, triggering biochemical and morphological events, such as depolarization of mitochondrial membrane potential and decreasing cell volume.

Conclusion Our data showed that vitamin K3 generates an oxidative imbalance in SiHa cells, leading to mechanisms that induce cell death by apoptosis.

Keywords Human Papillomavirus · Vitamin K3 · Apoptosis SiHa cells

Abbreviations

ANOVA	One-way analysis of variance
CCCP	Carbonyl cyanide 3- clorophenylhydrazone
DHR	Dihydrorhodamine 123
DIC	Differential interference contrast microscopy
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
FACS	Calibur flow cytometer
FSC-H	Forward light scatter
H2DCF-DA	2′,7′-dichlorodihydrofluorescein diacetate
HPV 16	SiHa cells
HPV	Human papillomavirus

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IC50	50 % inhibitory concentration
IC90	90 % inhibitory concentration
IV	Index of variation
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	Optical density
PI	Propidium idodite
PSN	Penicillin–streptomycin–neomycin antibiotic
RFU	Relative fluorescence units
Rh123	Rhodamine123
ROS	Reactive oxygen species
VERO	Cercopithecus aethiops
$\Delta\Psi m$	Mitochondrial membrane potential assay

Introduction

Cervical cancer is characterized as a major public health problem. According to the latest global estimates for the year 2012, cervical cancer is the fourth most common cancer among women, with 527,000 new cases [1]. The human papillomavirus (HPV) high carcinogenic risk genotypes 16 and 18 are the most prevalent in about 70 % of all cases of cervical cancer [2, 3].

The progression of HPV-associated cervical cancer involves the integration of the HPV genome into the DNA of the cervical cells, leading to the expression of two viral proteins, E6 and E7 [4]. These two proteins play a central role in the growth, proliferation, and apoptosis suppression, leading to the tumor development. Thus, inhibition of these proteins might be an alternative therapeutic mean for the treatment of HPV-cervical cancer [5]. It is known that E6 and E7 proteins are rapidly degraded by the ubiquitin–proteasome system [6, 7], which activity are enhanced in oxidative stress condition.

Numerous studies have been conducted, demonstrating the broad spectrum of action of vitamin K3 also known as menadione (2-methyl-1,4-naphthoquinone). These studies showed the anticancer effect of vitamin K3 for different types of cancer cells, such as neuroblastoma cell line [8], breast cancer [9], leukemia [10], and lung cancer [11]. This effect of vitamin K3 has been investigated in vivo and in vitro, and the results suggest that vitamin K3 may induce apoptosis via redox-cycling of the quinone structure to produce reactive oxygen species (ROS) [12, 13].

Therefore, the degradation of HPV proteins E6/E7 and the effect of vitamin K3 on cancer cell death seem to depend on a unique mechanism that involves the increase in reactive oxygen species (ROS) production. Based on that, our aim was to evaluate the antiproliferative effect and the mechanism of action of vitamin K3 against cervical epithelial cells transformed by HPV 16 (SiHa cells). Our data showed that vitamin K3 induces cellular redox impairment in SiHa cells, triggering signaling events that induce cell death by apoptosis.

Materials and methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin–neomycin (PSN) antibiotic, trypsin, dihydrorhodamine 123(DHR), actinomycin D, ethylenediaminetetraacetic acid (EDTA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA), and all other chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Propidium iodide (PI) was obtained from Invitrogen (Eugene, OR, USA). All of the other reagents were of analytical grade.

Vitamin K3

The vitamin K3 was acquired through Sigma-Aldrich (St. Louis, MO, USA) and kept at room temperature. Vitamin K3 solutions were prepared in DMSO and diluted in culture medium, so that the DMSO concentration did not exceed 1 % in the experiments. For each assay, a new drug dilution was performed. The concentrations of vitamin K3 used in the assays were based on the IC50 (50 % inhibitory concentration) and IC90 (90 % inhibitory concentration) values.

Cell culture

Cervical neoplasic epithelial cells (SiHa cell, HPV-16 positive) donated by Dr. Luísa Lina Villa, ICESP-USP, São Paulo, Brazil, and African green monkey kidney Cercopithecus aethiops ATCC CCL-81(VERO) were grown in DMEM medium supplemented with 5000 μg streptomycin and 125 μg amphotericin B and 10 % FBS at 37 °C in CO_2 incubator. After confluence and the cell monolayer formation, cells were trypsinized for 1 min, resuspended in DMEM containing 10 % FCS, and transferred to new plastic bottles.

Cell viability assay

Cell viability was evaluated in SiHa cells (5.0×10^5 cells/ml) and VERO cells (2.5×10^5 cells/ml), and incubated in DMEM supplemented with 10 % fetal bovine serum and

antibiotics (penicillin, streptomycin, and amphotericin B) at 37 °C in a 5 % CO_2 air mixture. The monolayer obtained after 24 h was treated with different concentrations ranging from 0.5 to 16 μM vitamin K3 for 48 h for SiHa cells and 72 h for VERO cells at 37 °C with 5 % CO_2. The cell viability was evaluated using the method 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), used to assess the ability of living cells to reduce MTT in insoluble formazan crystal violet. Intracellular formazan crystals were solubilized with DMSO, and the absorbance of the solution was measured at 570 nm in a spectrophotometer (Bio Tek—Power Wave XS). The percentage of viability was calculated as optical density (OD) of the cultures with treatment/DO control sample × 100 %.

Treatment types

On the basis of MTT results, two different doses IC50: 10.85 μM and IC90: 21.70 μM of vitamin K3 were selected for further experiments. Control groups received only media without any vitamin K3 treatment. Vitamin K3-treated cells were incubated for 48 h throughout all the experiments.

Detection of total reactive oxygen species

ROS production was evaluated during exposure of cells SiHa (5.0×10^5cells/ml) to 10.85 and 21.70 μM vitamin K3 using 2′,7′-dichloro dihydro fluorescein diacetate (H2DCF-DA) as a detector of intracellular ROS. H2DCF-DA can be converted by intracellular esterase into H2DCF, which is oxidized by ROS to highly fluorescent DCF. The cells were washed in Hankes' Balanced Salt Solution (HBSS) and incubated with 10.0 mM DCFH-DA in HBSS in 5 % CO2 at 37 °C for 30 min. Then, the cells were washed and incubated with HBSS buffer with iron free or iron supplemented medium. Afterward, the SiHa cells were treated with vitamin K3 for 48 h at 37 °C, and washed with HBSS and read. The fluorescence intensity was measured with a Varian fluorescence spectrophotometer with an excitation wavelength of 500 nm and emission detection at 529 nm. The arbitrary units, relative fluorescence units (RFU), were based directly on fluorescence intensity.

Mitochondrial membrane potential assay

Mitochondrial membrane potential was evaluated in SiHa cells (5.0×10^5cells/ml) treated with 10.85 and 21.70 μM vitamin K3 for 48 h at 37 °C in CO_2 incubator, using the fluorescent probe Rh123, which accumulates within mitochondria. Afterward, the cells were trypsinized, washed, and incubated with 5 mg/ml Rh123 for 15 min. CCCP (100.0 mM) was used as a positive control. The data acquisition and analysis were performed using an FACS Calibur flow cytometer (Becton–Dickinson, Rutherford, NJ, USA) equipped with Cell Quest software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA, USA). A total of 10,000 events were acquired in the region that was previously established as the one that corresponded to the cells. Alterations in Rh123 fluorescence were quantified using an index of variation (IV) obtained from the equation (MT−MC)/MC, in which MT is the median fluorescence for the treated and MC is the median fluorescence for the control. Negative IV values correspond to the depolarization of the mitochondrial membrane.

Microscopy analysis

Morphology was evaluated in SiHa cells (5.0×10^5 cells/ml) seeded in six-well plates with 10.85 and 21.70 μM vitamin K3 after 48 h. The cells were observed under a differential interference contrast (DIC) microscopy (Axiscope plus 2, Zeiss, Germany) and compared with the control group.

Cell volume determination

Cell volume was evaluated in SiHa cells (5.0×10^5 cells/ml) treated with 10.85 and 21.70 μM vitamin K3 for 48 h at 37 °C in CO_2 incubator. After trypsinization, the cells were collected by centrifugation, washed twice, resuspended in PBS, and analyzed using fluorescence-activated cell sorting and a FACS Calibur flow cytometer. Actinomycin D (20.0 mM) was used as a positive control. A total of 10,000 events were acquired in the region that was previously established as the one that corresponded to the cells. Histograms were generated, and the analysis was performed using Cell Quest software; forward light scatter (FSC-H) represents the cell volume.

Cell membrane integrity assay

The cell membrane integrity was evaluated in SiHa cells (5.0×10^5 cells/ml) treated with 10.85 and 21.70 μM vitamin K3 for 48 h at 37 °C in CO_2 incubator using PI, a probe that binds to DNA into cells ruptured membrane. Thereafter, the cells were washed and incubated with 0.2 mg/ml PI for 10 min to verify cell membrane integrity. Digitonin (40.0 mM) was used as a positive control. Data acquisition and analysis were performed using an FACS Calibur flow cytometer equipped with Cell Quest software. A total of 10,000 events were acquired in the region that was previously established as the one that corresponded to the cells. Alterations in the fluorescence of PI were quantified as the percentage of increase in the fluorescence compared with the control (untreated cells).

Statistical analysis

Statistical analysis of results (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, CA, USA) was performed by one-way analysis of variance (ANOVA) followed by the Newman–Keuls multiple comparison test or by two-way ANOVA when the effects of two different factors were studied in the same experiment. The results were obtained from three independent experiments, and the bar represents the mean ± S.E. *p-values of <0.05 were considered significant.

Results

Vitamin K3 induces antiproliferative effects in SiHa cells

The literature provides some evidences of the cytotoxic effect of vitamin K3 on cancer cells [8–11]. Thus, we first evaluated the SiHa cells viability after 48 h of treatment to determine the IC50 and IC90 of vitamin K3 using MTT assay, a standard method to assess cell viability. Figure 1 shows that vitamin K3 inhibit SiHa cells proliferation in all tested concentration with an IC50 of 10.85 μM and an IC90 of 21.70 μM. For VERO cells, the cytotoxicity of vitamin K3 was lower in all tested concentrations with a CC50 (50 % cytotoxic concentration) of 18.4 μM.

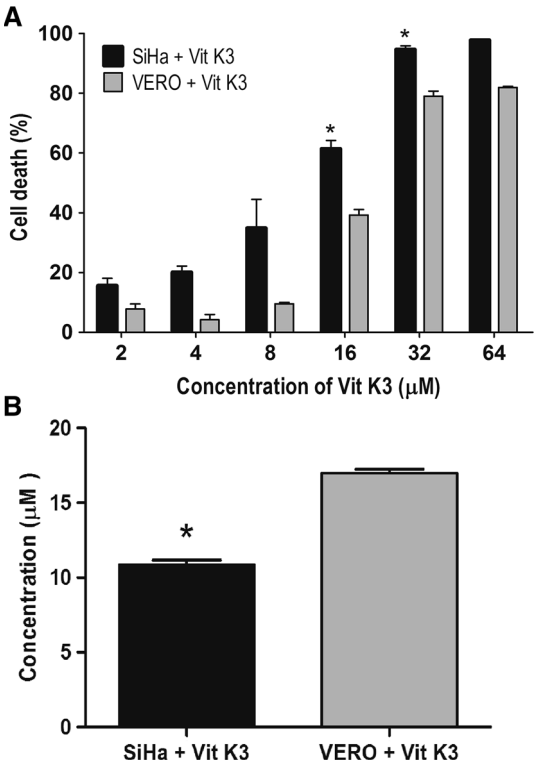

Fig. 1 Analysis of cytotoxic effect of vitamin K3. Cells were cultured in the presence of various concentrations of vitamin K3 (2–64 μM/mL) for 48 h, and cell proliferation was measured by MTT assay. **a** SiHa (5.0×10^5cells/ml) and VERO (2.5×10^5 cells/ml) cells viability in vitamin K3-treated cells. **b** IC50 and CC50 values of vitamin K3 on SiHa and VERO cells, respectively. *$p \leq 0.05$ indicates significant difference relative to the vitamin K3-treated VERO cells

Vitamin K3 increases the production of total ROS in SiHa cells

Vitamin K3 has been described to induce tumor death by increasing the cellular formation of ROS [12, 13]. To understand that, we evaluated the total ROS production in SiHa cells after 48 h of vitamin K3 treatment using flow cytometry, in which the non fluorescent H2DCF-DA is oxidized by ROS, producing fluorescent DCF. Figure 2 shows that vitamin K3 induced a significant increase 50.1 % in intracellular oxidant species formation by SiHa cells at both, IC50 and IC90 treated cells compared with the control group.

Vitamin K3 induces depolarization of mitochondrial membrane potential (ΔΨm) in SiHa cells

Mitochondria are cytoplasmic organelles with a remarkable function of produce ATP through the respiratory chain and regulate cellular metabolism controlling the mitochondrial ROS production, the mitochondria membrane potential, and the apoptosis cell death [14]. Here, we evaluated the ΔΨm in vitamin K3-treated SiHa cells stained with Rh 123, in which Rh123 accumulates within normal mitochondria using flow cytometry. Histograms of total Rh123 fluorescence showed a decrease in fluorescence intensity,

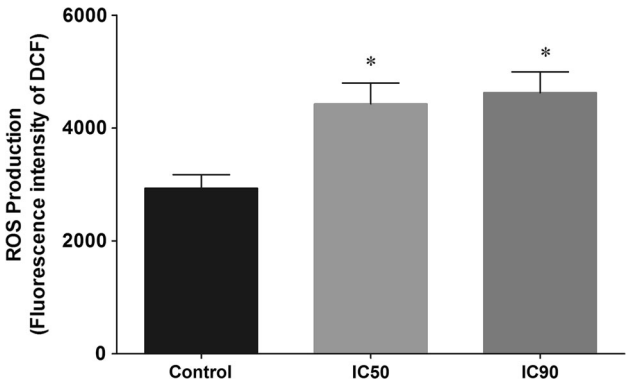

Fig. 2 Total ROS production in vitamin K3-treated SiHa cells for 48 h. Total ROS production was measured in cells stained with H_2DCF-DA and analyzed by fluorescence intensity of DCF. SiHa cells (5.0×10^5 cells/ml) were treated with IC50: 10.85 μM and IC90: 21.70 μM vitamin K3 for 48 h. *$p \leq 0.05$, significant difference relative to the control group (untreated cells)

Table 1 Mitochondrial membrane potential assay in SiHa cells treated with vitamin k3 for 48 h and stained with Rh123

SiHa		
μM	Median	IV[a]
Control	1893.41	0.00
10.85	90.99*	−0.95
21.70	89.77*	−0.97

[a] *IV* (M_T–M_C), where M_T corresponds to the median fluorescence for treated SiHa cells, and M_C to that median fluorescence for control SiHa cells

* $p \leq 0.05$, significant difference relative to the control group (untreated cells)

indicating that the mitochondria were depolarized in SiHa cells treated with 10.85 and 21.70 μM of vitamin K3 for 48 h. As shown in Table 1 and Fig. 3, the reductions of ΔΨm were 95.19 and 95.26 % for 10.85 and 21.70 μM of vitamin K3, respectively, compared with the control group. The positive control, CCCP, induced 35.6 % the reductions changes in mitochondrial membrane potential. These data show that vitamin K3 causes the loss of ΔΨm, which is essential for mitochondria ATP production.

Vitamin K3 induces morphological changes in SiHa cells related to apoptosis

Mitochondrial function is also important to maintain the cell homeostasis, such as the regulation of the cell volume, and cellular morphology [15]. As our data showed that vitamin K3 causes the loss of ΔΨm, we then evaluated the effect of vitamin K3 on SiHa cell morphology, using phase microscope of interference contrast difference (DIC). As expected, vitamin K3 in both tested concentrations caused morphological changes in SiHa cells closely related to apoptotic-like cell death, reflected here by round-shaped, plasma membrane blebs, and shrink cells (arrow) (Fig. 4c), compared with the control group (Fig. 4a).

Vitamin K3 decreases SiHa cells volume

The morphological changes induced by vitamin K3 in SiHa cells evidenced cell morphology alterations characteristic of apoptosis. Thus, to confirm it, we next evaluated the effect of vitamin K3 on SiHa cells volume using flow cytometry. As expected, our results shown that vitamin K3 induced a decrease in cell volume on SiHa cells in both concentrations tested after 48 h. These reductions were 52 and 43 % for IC50 and IC90 (Fig. 5a, b), respectively, compared with the control group. The positive control, actinomycin D, is also shown with a decreased SiHa cell volume of 36 % (Fig. 5c).

Vitamin K3 does not alter SiHa cells membrane integrity

The previous data prompt us to evaluate the effect of vitamin K3 on SiHa cells membrane integrity using PI, a probe that is excluded from healthy cells but binds to DNA in ruptured membrane cells. Figure 6 shows that vitamin K3 does not affect the membrane integrity of SiHa cells, even at IC 90 dose, compared with the control group. According to the histograms, both concentrations (IC50 and IC90) display the same PI fluorescence intensity compared with the control group (Fig. 6a, b), indicating no alterations of cell membrane integrity. Bright fluorescence was observed with the positive control, digitonin (data not shown). Membrane integrity is also a hallmark of apoptosis [16].

Discussion

Cervical cancer is one of the most common types of cancer with a high mortality rate among women, being characterized as a major public health problem [17]. Treatment for this type of pathology is typically invasive. Thus, new assets and combinations of drugs are constantly studied [9]. Platinum-based chemotherapy in combination with radiotherapy or surgery is now mainly used, but the efficacy is limited, especially in advanced-stage disease [18, 19]. Nowadays, the search for antitumor drugs of high efficacy and low toxicity for the treatment of cervical cancer is challenging. The effect of vitamin K3 has been investigated in various types of cancer cells, such as neuroblastoma cell line [8], breast cancer [9], leukemia [10], and lung cancer [11]. Several studies suggest that this vitamin may induce apoptosis through different biochemical pathways, and thereby, signaling mechanism of cell death induced by treatment with vitamin K3 has been detailed [9, 10, 12]. However, the role of vitamin K3 in cervical cancer and the precise mechanism of action had not been yet elucidated. Therefore, we studied the effect of vitamin K3 on human cervical cancer SiHa cells (HPV 16), and herein, we described some of the biochemical action of vitamin K3 on HPV 16. For this, we investigated the production of total reactive oxygen species; mitochondrial membrane potential; cell morphology; cell volume; and cell membrane integrity.

It is known that biological behavior of malignant tumor cells includes disorderly proliferation and distant metastasis [20]. Vitamin K3 induced cytotoxic effects in SiHa cells, inhibiting cell proliferation. These results were expected, because other authors have also been published similar cytotoxic activity of vitamin K3 against different types of cancer cells [8–10]. To better understanding of the

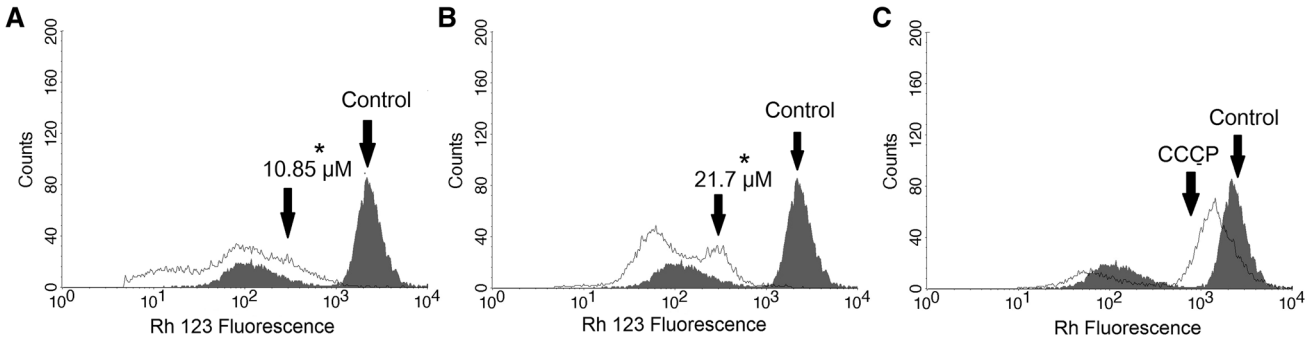

Fig. 3 Mitochondrial membrane potential assay in SiHa cells treated with vitamin K3 for 48 h and stained with Rh123, which accumulates within mitochondria and was analyzed by flow cytometry. **a** SiHa cells (5.0×10^5cells/ml) treated with IC50: 10.85 µM. **b** SiHa cells (5.0×10^5cells/ml) treated with IC90: 21.70 µM. **c** SiHa cells treated with 100 mM CCCP for 48 h. *Arrows* correspond to tested concentration. $*p \leq 0.05$ indicates significant difference relative to the control group (untreated cells)

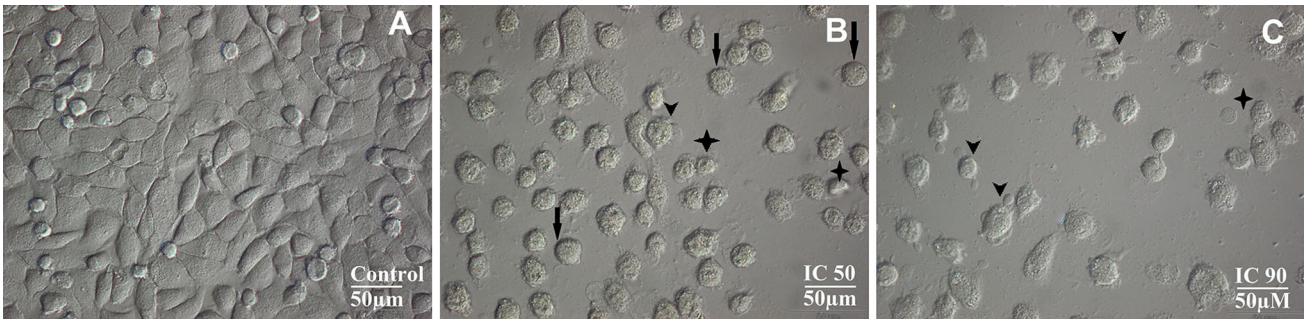

Fig. 4 Morphological changes of vitamin K3-treated SiHa cells (5.0×10^5 cells/ml) after 48 h using microscope of interference contrast difference (×400). **a** Untreated SiHa cells. **b** SiHa cells treated with IC50: 10.85 µM. **c** SiHa cells treated with IC90: 21.70 µM. (*arrow*) *round-shaped*; (▼) plasma membrane blebs; and (*plus*) shrink cells. *Scale bar* 50 µM

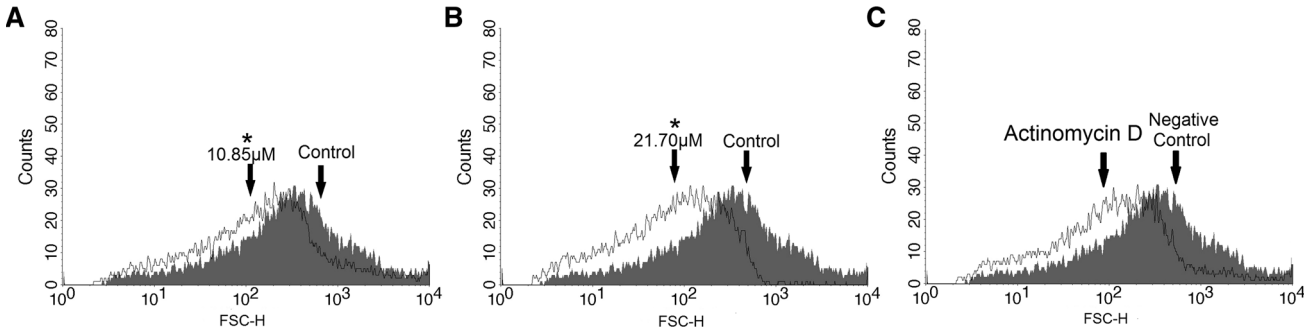

Fig. 5 Cell volume in vitamin K3-treated SiHa cells (5.0×10^5 cells/ml) after 48 h using flow cytometry. **a** SiHa cells treated with IC50: 10.85 µM. **b** SiHa cells treated with IC90: 21.70 µM. **c** SiHa cells treated with actnomicyn D 20.0 mM. FSC-Height was considered as a function of cell size. *Arrows* correspond to tested concentrations of vitamin K3. $*p \leq 0.05$, significant difference relative to the control group (untreated cells)

likely mechanism of cytotoxic effect of vitamin K3 in SiHa cells, we performed some biochemical redox analysis of vitamin K3-treated SiHa cells.

Vitamin K3 was able to modify the cellular redox equilibrium, increasing ROS production in SiHa cells. Differently of healthy cells, tumor cells can easily endure the high levels of ROS, avoiding death and continuously inducing the growth through changes in the metabolism, such as expression of detoxification proteins [21]. We believe that vitamin K3 is modulating the production of ROS in a way that the cellular ROS concentration overcomes the capacity of tumor to keep the redox state within a limit that promotes cell proliferation. In addition, there are evidences that high ROS level mediated the

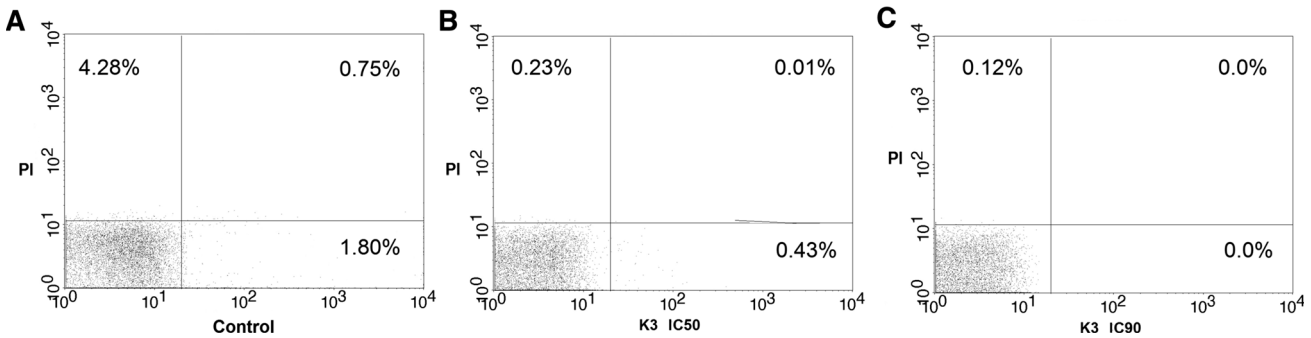

Fig. 6 Cell membrane integrity assay in vitamin K3-treated SiHa cells (5.0×10^5cells/ml) after 48 h in cells stained with PI a probe that binds to DNA in ruptured membrane cells using flow cytometry. **a** Untreated SiHa cells. **b**, **c** SiHa cells treated with IC50: 10.85 μM and IC90: 21.70 μM. Percentage of PI-stained positive cells is shown in the *upper right* and *left quadrants*

ubiquitin proteasome system activation, leading to E6/E7 degradation, culminating with HPV-immortalized cells death [6, 7].

The literature shows that mitochondria are organelles with cytoplasmic regulatory function of cellular metabolism and in cancer cells that metabolism can be altered. In addition, mitochondria are organelles responsible for the synthesis of adenosine triphosphate, as well as being involved in the formation of ROS/RNS [22]. Vitamin K3 induced mitochondrial membrane depolarization in SiHa cells. Although the "Warburg effect" [23] states that cancer cells have an injured mitochondria and use the aerobic glycolysis instead oxidative phosphorylation, recent studies have shown that cancer cells present distinct energy metabolism and might depend on oxidative phosphorylation to produce energy [24–26]. For example, in the cervical epithelial cancers (SiHa and Hela), the ATP synthesis is mainly mitochondrial [27, 28]. Thus, many antitumor drugs have been described with the mitochondria as their main worthy target [29], including vitamin K3 [9, 30].

Vitamin K3 also induced morphological changes in SiHa cells related to apoptosis [31]. Apoptosis is a programmed cell death with orchestrated biochemical events that controls cells proliferation [16]. Increase in apoptosis signaling pathway may induce tumor regression [32].

Our results demonstrate that vitamin K3 might be triggering biochemical cell events that activate the signaling pathways for apoptosis cell death. We believe that the effect of vitamin K3 in SiHa cells is related to the redox-cycling of the quinone structure to produce ROS [12, 13]. The increase in ROS might induce the degradation of E6 and E7 proteins, which play a central role in the progression of HPV-associated cervical cancer inducing growth, proliferation, and apoptosis suppression. Thus, our data support further studies, concerning the effect of vitamin K3 on the expression of E6 and E7 proteins, which might be an alternative therapeutic mean for the treatment of HPV-cervical cancer [5].

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Compliance with ethical standards

Ethical standards This study was funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes), Financiadora de Estudos e Projetos, Fundação Araucária. Programa de Pós Graduação em Biociências e Fisiopatologia da Universidade Estadual de Maringá, and Complexo de Centrais de Apoio a Pesquisa–UEM.

Conflict of interest All authors have no conflict of interest.

Human participants This article does not contain any studies with human participants or animals performed by any of the authors.

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