Ascorbic Acid Modulates Doxorubicin and Cyclophosphamide-Induced Cytogenetic Damages in Sarcoma 180 Cells

ORIGINAL RESEARCH

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Abstract

Cancer is a public health global problem. Cyclophosphamide (CPA) and Doxorubicin (DOX) are used in chemotherapy, especially by generating reactive oxygen species. The clinical use of ascorbic acid during chemotherapy also raises several controversies due to its antagonistic effects on antineoplastic agent. In this sense, this study aims to evaluate the effects of ascorbic acid (2 µg/mL) on the modulation of cytogenetic damage induced by CPA (20 µg/mL) and DOX (2 µg/mL), as well as their interaction (AC protocol) on Sarcoma 180 tumor cells. Cytogenetic damage classified as apoptosis, necrosis, micronuclei, buds and nucleoplasmic bridges were linked to the Cytokinesis-Block Micronucleus Assay application. CPA and DOX induce significant (p<0.05) increases in apoptosis, necrosis and micronuclei in the cell types tested. The damage on Sarcoma 180 cells was modulated by ascorbic acid with percentage modulation in more than 70% of CPA relative to apoptosis and micronuclei, and 32% to necrosis. The damage of DOX has been modulated by 70% to apoptosis, 40% to necrosis, rather than micronuclei, whereas, in AC protocol, modulations were observed by 52% to apoptosis and 32% and 40% to necrosis and micronuclei, respectively. There was no significance in relation to the nuclear buds and nucleoplasmic bridges. In addition, ascorbic acid did not show any effect. These results are other studies, which indicate hazards to chemotherapy effectiveness due to the antioxidant effects

of ascorbic acid in the modulation of oxidative stress to promote major cytogenetic damage that is important to tumor regression.

Introduction

Cancer is a complex and multifactorial disease with epidemiological profile. Its etiology is assumed as genetic mutations that cause unlimited capacity of cell proliferation, loss of response to growth inhibition factors, evasion of apoptosis (programmed cell death), opportunities to invade other body tissues (metastases), and production of new blood vessels (angiogenesis) [1, 2].

The therapeutic modality for cancer treatment is drug therapy, such as chemotherapy. It is generally known for its partial selective destruction of tumor cells, but causing several side effects to patients and damage to normal cells [3, 4]. The identification of the variety of responses to cancer therapy requires knowledge of variables. It includes concomitant medications, which may alter the metabolism and pharmacokinetics of chemotherapy, and pointing to drug interactions [5, 6].

There are several therapeutic modalities for cancer, including the protocol associating doxorubicin (DOX) plus cyclophosphamide protocol (AC). The most common doses of these drugs are, respectively, 60/600 (mg/m$^{2)}$) every 21 days [7]. Cyclophosphamide (CPA) is a cytotoxic agent with immuno action [8, 9] and alkylating to the genetic material, which induces apoptosis by free radicals [10, 11].

On the other hand, some researchers have determined that several factors may influence cellular DOX toxicity, most notably the expression of the membrane transporter P-glycoprotein/MDR1 (P-

gp) and the generation of reactive oxygen species (ROS) and free radicals through the redox cycle of doxorubicin, as well as inhibition of topoisomerase II [12,13].

Most patients in chemotherapy make supplementation with vitamins, especially, vitamin C, due to its high antioxidant capacity, which may cause antagonistic effects to antineoplastic and neutralize some carcinogens [14,15]. Antioxidants such as vitamin C compose the human diet [16], which not only sequester free radicals, but also regulate DNA repair enzymes and post-transcriptional factors [17]. Vitamin C (ascorbic acid), a cofactor for the alpha-ketoglutarate dioxygenase, is important for collagen formation, as well as transcriptional factor, which regulates genes involved in tumor growth and apoptosis [18, 19].

In vitro studies of human MCF-7, vitamin C exposure causes attenuation of antineoplastic, it may affect the therapeutic response [15]. There are also reports that indicate high doses of ascorbic acid, increasing apoptosis in Sarcoma 180 cells on mice co-treated with paclitaxel, enhancing the effects of anticancer [20].

However, there are numerous controversies about the role of antioxidant/pro-oxidant, ascorbic acid (AA), and its use as antioxidants during chemotherapy, half-life period of antineoplastic [21-24]. Thus, this study aims to contribute to the understanding of anticancer in association with ascorbic acid in front of cytogenetic damage in the Sarcoma 180

model, as well as the analysis of damage in lymphocytes of mice with the application of cytokinesis-blocked micronucleus assay.

Materials and Methods

Chemicals

The antineoplastic agents, cyclophosphamide (CPA) and doxorubicin (DOX) were purchase from Eurofarma® Laboratories and prepared by dilution with sterile 0.9% saline solution to a final concentration of 20 µg/mL and 2 µg/mL, respectively. The combination of both antineoplastic agents was prepared in the same way, keeping the same concentrations. The ascorbic acid (Sigma, St. Louis, MO) was solubilized in 50 mM phosphate buffer (pH 7.4) till the final concentration of 2 µg/mL. This concentration was predetermined because it may not produce pro-oxidant effects, as reported by Putchala [25] and Bartel [26].

Primary culture of Sarcoma 180 cells

Cell cultures of Sarcoma 180 (S180) were performed in RPMI 1640 medium, supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin, at 37°C with 5% CO_2. Ascite-bearing female mice between 7 and 9 days post inoculation were sacrificed by cervical dislocation and a suspension of S180 cells was harvested from the intraperitoneal cavity under aseptic conditions [27]. The investigational protocols were approved by the local Ethical Committee on Animal Research (Process No. 081/2014) and they are in accordance with Brazilian (COBEA - Colégio Brasileiro de Experimentação Animal) and international guidelines on the care and use of experimental animals (Directive 2010/63/EU of the European Parliament and of the Council). The suspension was centrifuged at 500 X g for 5 min to obtain a cell pellet and washed three times with RPMI medium. Cell concentration was adjusted to

0.5×10^6 cells/mL in supplementing RPMI 1640 medium flasks, and incubated with concentrations of the CPA (20 µg/mL), DOX (2 µg/mL) and ascorbic acid (2 µg/mL).

Cytokinesis-block micronucleus assay (CBMN)

For this study, we followed criteria for the identification of binucleate cells and CBMN assay parameters as described by Fenech [28], with some adaptations. Briefly, S180 cell suspension (0.5×10^6/mL) was added in a culture flask of supplementing RPMI 1640 medium (20% fetal bovine serum), antibiotics (Sigma, St. Louis, MO) and phytohemagglutinin A (Gibco, Grand Island, NY). Each reaction flask was then treated with antineoplastic agents separately, and the combination of both was treated in a flask marked AC treatment. In addition, AA was added in the co-treatment. Cells were incubated for 44 h at 37 ± 1 °C. After incubation, 6 µg/mL of cytochalasin B (Sigma, St. Louis, MO), they were added to the culture flask. Next, the flasks were returned to incubation for additional 28 hours. At the end of incubation phases, the cultures were transferred to Falcon tubes and centrifuged at 800 rpm for 5 minutes. Then, the supernatant was removed and the cell pellets were gently shaken, added 5 mL of fixative (methanol: acetic acid 5:1) and 3 drops of formaldehyde, the tubes were centrifuged again. The procedure was repeated twice with a fixative solution (3:1), and without formaldehyde. Finally, the supernatant was discarded and 2 to 4 drops of cell suspension were dripped on slides, which were stained with Giemsa 5% (exposure time of 7 minutes.)

The previously coded slides were examined in a blind test with an optical microscope at 1000X magnification, considering the cytogenetic damage presented at 1000 cells per slide in duplicate. Nuclear division cytotoxicity index (NDCI) was calculated with the equation: NDCI = (Ap + Nec + M1 + 2(M2) + 3(M3) + 4(M4))/N*, in which Ap

represents the number of apoptotic cells, Nec, the number of necrotic cells, M1-M4, the number of viable cells with 1-4 nuclei, and N^*, the total number of viable and nonviable cells scored, according to Fenech [29].

Trypan blue exclusion test

Cell viability was analyzed by the application of the Trypan blue exclusion test according to Renzi [30]. After 72 hours of treatment with the antineoplastic agent, as well as untreated cells, 90 µL of S180 cell suspensions were removed from culture and added to 10 µL of Trypan Blue to different treatment groups: CPA, DOX and AC. Non-viable cells were counted by its blue color, therefore, considered as dead cells. In contrast, viable cells did not present this color due to their ability to expel the trypan blue. Cell differentiation was observed under an optical microscope with a magnification of 40X increased with Neubauer chamber's assistance.

Cytogenetic modulatory effect of AA in S180 cells line

Values of percentage modulation (%M) of AA front cytogenetic damage induced by antineoplastic agents CPA and DOX, as well as the associated in the AC protocol were calculated according to the following formula: %M = (X-(Y+AA))/X*100, which "X" represents the values of cytogenetic damage induced by antineoplastic and "Y + AA" represents the values of cytogenetic damage induced by antineoplastic associated with AA.

Statistical analysis

For this purpose, it was used Graph Pad Prism 6.0 (Graph pad Inc., San Diego, CA). The differences between groups were analyzed using one-way analysis of variance (ANOVA), followed by Turkey's test. The calculated values are expressed as mean ± standard deviation (SD) and considered statistically significant with $p < 0.05$.

Results

Cell viability

S180 cell viability was performed after 72 h *ex vivo* exposure to CPA, DOX and AC. At the end, we applied the exclusion technique by Trypan blue. The treatment of S180 cells with CPA reduced cell viability significantly ($p < 0.05$) compared to baseline, however, cytogenetic events were significantly modulated ($p < 0.05$) by co-treatment with AA **(Figure 1)**. Similarly, DOX also reduced cell viability ($p < 0.05$), but these effects were not modulated by AA. Regarding the AC treatment, the results were consistent with the CPA that also inhibited cell viability compared to baseline, but modulated by AA ($p < 0.05$) **(Figure 1)**.

Figure 1: S180 cells exposed to antineoplastic and co-treated with AA. (Data represents the % of cell viability of 4 independent experiments. CPA: cyclophosphamide (20 µg/mL); DOX: doxorubicin (2 µg/mL); AC: DOX+CPA (2:20 µg/mL); AA: ascorbic acid (2 µg/mL). *p<0.05 compared to untreated cells; *a p<0.05 compared to CPA; *b p<0.05 compared to AC.).
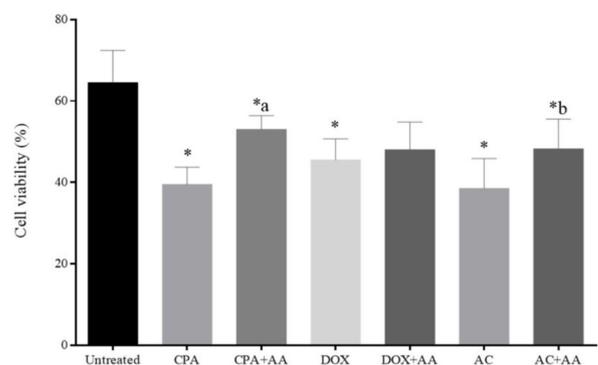
Evaluation of cytogenetic damage by CPA, DOX and AC on S180 cells cultures, and the effects of AA co-treatment

The CPA, indeed, ($p < 0.05$) induced apoptosis in S180 cultures in relation to the untreated baseline. However, the CPA+AA significance ($p < 0.05$) modulates

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Table 1. Cytogenetic damage induced by CPA, DOX and AC and modulatory effects of AA co-treatment in S180 cells line..

Treatment	Cytogenetic damages					NDCI
	Apoptosis	*Necrosis*	*Micronuclei*	*Nuclear bu^{d}s*	*Nucleoplastic bri^{d}ges*	
Untreated cells	47.5 ± 7.7	39.5 ± 3.5	0.50 ± 0.7	5.5 ± 3.3	2.5 ± 0.7	0.979 ± 0.12
CPA (µg/mL)	294.5 ± 17.6[a]	115.0 ± 2.8[a]	8.5 ± 2.1[a]	21.5 ± 7.7[a]	10.5 ± 0.7[a]	0.716 ± 0.06[a]
CPA+AA (µg/mL)	76.0 ± 8.4[b]	77.5 ± 3.5[b]	2.5 ± 0.7b	26.0 ± 1.4	7.5 ± 2.8	0.813 ± 0.14b
DOX (µg/mL)	279.5 ± 28.9[a]	93.5 ± 3.5[a]	18.5 ± 4.9[a]	9.5 ± 3.5	5.0 ± 2.5	0.683 ± 0.05[a]
DOX+AA (µg/mL)	80.0 ± 2.8[c]	56.0 ± 4.2[c]	14.5 ± 2.1	8.5 ± 2.9	4.5 ± 0.7	0.748 ± 0.10[c]
AC (µg/mL)	568.5 ± 34.6[a]	85.0 ± 7.0[a]	20.0 ± 1.4[a]	8.0 ± 4.2	6.5 ± 2.1	0.380 ± 0.17[a]
AC+AA (µg/mL)	297.0 ± 137.2[d]	58.0 ± 4.2[d]	12.5 ± 2.2[d]	7.5 ± 2.5	6.0 ± 4.2	0.494 ± 0.14[d]

Values are mean ± standard deviation of 2000 cells evaluated. CPA: cyclophosphamide; DOX: doxorubicin; AC: doxorubicin + cyclophosphamide; AA: ascorbic acid. NDCI: nuclear division cytotoxicity index. ANOVA-one-way followed by Tukey's test. [a]: $p<0.05$ compared to untreated cells. [b]: $p<0.05$ compared to CPA. [c]: $p<0.05$ compared to DOX; [d]: $p<0.05$ compared to AC.

apoptosis induced by treating the cells with CPA **(Table 1)**. Similarly, CPA induced significant ($p<0.05$) increases in necrosis of S180 cells, when compared to baseline. However, AA (2 µg/mL) has modulator ($p<0.05$) effect on the necrosis induced by CPA. For other cytogenetic damage, such as micronuclei, nuclear buds and nucleoplasmic bridges, the CPA significantly ($p<0.05$) induced the formation in S180 cells. Nevertheless, in association with ascorbic acid, only micronuclei was significantly ($p<0.05$) modulated **(Table 1)**.

DOX also induced significantly ($p<0.05$) apoptosis and necrosis. However, it did not induce nuclear buds and bridges (insignificant data), but induced mutagenicity by the significant ($p<0.05$) increasing in micronuclei frequency. We emphasize that the co-treatment with DOX+AA modulated ($p<0.05$) the apoptosis and necrosis effects of DOX **(Table 1)**.

The CPA combination with DOX (AC protocol), significant ($p<0.05$) induced apoptosis and necrosis of S180 cells. However, these effects were significantly ($p<0.05$) modulated by AA. Regarding to clastogenic and/or aneugenic effects of AC, the data were significant ($p<0.05$) for the formation of micronuclei and were modulated ($p<0.05$) by co-treatment with AA. Similar to DOX data from the

cytogenetic damage, the AC did not induce bridges and nuclear buds (insignificant data) **(Table 1)**.

In **Figure 2**, we present the overall photomicrograph profile of cytogenetic damage produced by antineoplastic, as well as co-treatment with AA. The cytotoxicity of antineoplastic CPA, DOX and AC, as well as the effects of AA co-treatment were evaluated by calculating the NDCI (nuclear division cytotoxicity index). The proportion of apoptosis and

Figure 2: Photomicrographic profile of effects produced by AA in S180 cells exposed to DOX, CPA and AC. (5% Giemsa staining, 100X. CPA: cyclophosphamide 20 µg/mL. DOX: doxorubicin 2 µg/mL. AC: DOX+CPA (2:20 µg/mL); AA: ascorbic acid 2 µg/mL. AP: apoptosis. NC: necrosis. MN: micronuclei. BT: nuclear bud. PT: nucleoplasmic bridge.)
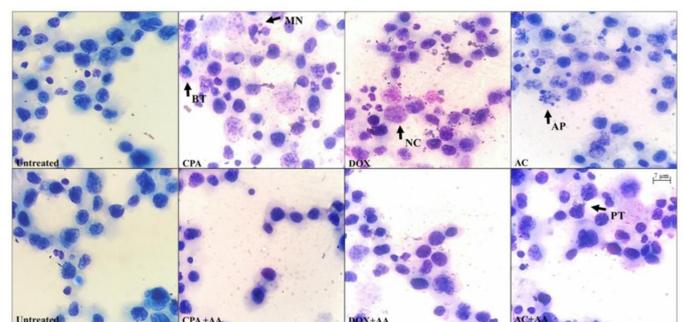
necrosis reflects the cellular sensitivity of the tested antitumor treatment. The CPA, DOX and AC showed cytotoxicity **(Table 1)**, with the significance of baseline damage, but co-treatment with AA modulated the cytotoxicity induced by antineoplastic treatment.

Evaluation of cytogenetic modulatory effect of AA in S180 cells line

The data presented in **Table 2** were plotted into the modulation percentage (%M) for cytogenetic alterations of the apoptosis, necrosis, micronuclei and bridges induced by CPA, DOX and AC. Effectible ascorbic acid had modulated apoptosis induced by CPA with 74% (74.13 ± 0.85); however, for necrosis it was by 32% (32.04 ± 0.81), which indicates a better performance for modulating apoptosis. Similarly, AA had modulated aneugenic and clastogenic effects induced by CPA in S180 cells with 70% (70.25 ± 4.71) inhibition of micronuclei. It reports that the bridges induced by CPA were not modulated by AA.

Table 2. Cytogenetic modulatory effect of AA in S180 cell line.

Cytogenetic damage	Modulation percentage (%M)		
	CPA+AA	*DOX+AA*	*AC+AA*
Apoptosis	74% (74.13 ± 0.85)	70% (70.25 ± 1.25)	52% (52.2 ±7.54)
Necrosis	32% (32.04 ± 0.81)	40% (39.75 ± 1.25)	32% (319 ± 1.25)
Micronuclei	70% (70.25 ± 4.71)	NM	40% (39.2 ± 4.27)

Values are mean ± standard deviation. Data are presented by modulation percentage (%M) calculated with the equation: $\%M = (X-(Y+AA))/X*100$, where %M represents the modulation percentage of AA. X: represents the values of cytogenetic damage induced by antineoplastic (see Table 1). Y+AA: represents the values of cytogenetic damage induced by antineoplastic associated with ascorbic acid (AA). AA: ascorbic acid. CPA: cyclophosphamide; dOX: Doxorubicin. AC: doxorubicin + cyclophosphamide. NM: no modulation.

Effectible DOX-induced apoptosis had modulated by AA with more than 70% (70.25 ± 1.25) in a similar way to CPA. As observed with CPA, AA modulation percentage compared to necrosis was lower (40%, 39.75 ± 1.25). The mutagenicity (increased in micronuclei) induced by DOX was not modulated by AA. As it was noticed in **Table 2**, the modulation percentage of cytogenetic effects induced by AC was lower than those observed for CPA and DOX. However, AA modulated about 52% (52.2 ± 7.54) for apoptosis, and 32% (31.9 ± 1.25) for necrosis. The micronuclei were modulated by 40% (39.2 ± 4.27) with AA.

Discussion

Although the well described benefits of antioxidants like ascorbic acid in the prevention of cancer, there are still doubts about their interference in chemotherapy. Antioxidants capture the superoxide radical, and thus, deplete the supply of hydrogen peroxide intracellular, which induces cancer. Moreover, it causes elimination of hydroxyl radicals, thus the use of antioxidants may reduce the effects of chemotherapy or radiation, due to the elimination of free radicals, since most of the radiation and antitumor drugs act precisely in the production of free radicals to struggle against cancer cells [31].

To get acquainted to the action mode of an antitumor drug is essential for the development of new drugs in this line. It is believed that the antitumor activity, mediated by certain drugs, can induce DNA damage. Thus, it may trigger the apoptosis. The selection is mainly based on the specific chemotherapeutic cytotoxicity for the tumor cell type and relatively low toxicity to normal cells and tissues. However, toxicity to normal cells has a high clinical, particularly, when malignant cells become resistant to chemotherapy, which results from factors, such as, individual variation, tumor genetic heterogeneity in tumors and tumor evolution [32].

Apoptosis is a process of cellular responses to multiple stresses, including DNA damage, and it is considered as one of the bases for therapeutic

response [33]. In our studies, CPA induced apoptosis in S180 cells **(Table 1)**. These data point out the CPA action on neoplastic cells. Reactive oxygen species play a central role in the mechanisms involved in redox signaling and control of apoptosis in the cytosol and mitochondria to release cytochrome c, which are the initial events of apoptosis. The antioxidant enzymes, such as, glutathione, play a role in the catalytic site of the cysteine that can activate and inactivate caspases in the control of apoptosis [34].

A major goal of therapies against cancer is killing cancer cells. Several ways of cell death, including apoptosis and necrosis, can lead to the cancer cells death. Even with the great variability of cancer, studies indicate that resistance to apoptosis is one of the most striking features of most malignant tumors. The drugs used on the chemotherapy result in DNA damage and response to stress. They can also lead to apoptotic death in some cancer cells. CPA is an important antineoplastic used to treat cancer, because it induces a large number of apoptosis [35, 36], which can be related to ROS action [37].

However, antioxidants protect cells against DNA damage because they are able to sequester free radicals [38]. In addition, these free radicals can regulate DNA in order to repair enzymes, and post-transcriptional factors [19]. High levels of diets with antioxidants and/or supplementations may interfere in the action of pro-oxidants chemotherapeutic agents [39, 40]. The combination of co-treatment of AA at a concentration of 2 µmol/mL with CPA, as well as in AC cause a modulation of the apoptosis induced by antineoplastic treatment in about 74% and 52%, respectively **(Table 2)**.

Heaney [41] reported that *in vivo* ascorbic acid reduces the cytotoxic effects of DOX 1 mg/kg. In addition, ascorbic acid attenuates antineoplastic drug like 5-fluorouracil (150 mg/kg) induced gastrointestinal toxicity in rats by modulating the expression of inflammatory mediators. The inhibitory

potential of AA may be due to the modulation of oxidative stress, and also activation of redox sensitive transcription factor [42]. This interference was also observed in this study, which the apoptosis was reduced in CPA co-treatment with AA compared to a treatment with only a CPA, since this is an alkylating agent that induces apoptosis in rapidly dividing cells [43].

The results of Shatzer's study [44], who also demonstrated that pharmacological concentrations of AA are highly antagonistic to bortezomib compared to *in vitro* B cell positive for Epstein-Barr virus. Bortezomib is an inhibitor of the 26S proteasome, such as, CPA, thus, it shows antitumor activity in a variety of malignant tumor [45].

In vitro studies with human MCF-7, AA exposure causes attenuation of antineoplastic activity and may affect the therapeutic response [15]. There are also reports indicating that AA can enhance the effectiveness of chemotherapeutic agents that can reduce the cytotoxicity of anticancer drugs, and also may prevent free radicals and neutralize some carcinogens [14,46,47]. The identification of the variety of responses to cancer therapy requires knowledge of variables, including concomitant medications, which may alter the metabolism and pharmacokinetics of chemotherapy. Clinical studies showed drug interactions [6].

Several epidemiological studies investigating the effects of AA against breast cancer link many inconsistent results, but, reports indicate that it can also reduce the risk of this type of cancer due to its antioxidant action for protection of DNA damage by neutralizing the free radicals and initiation of carcinogenesis [48].

DOX is one of the most effective antitumor drugs, but its clinical use is limited due to some adverse effects, such as, cardiotoxicity induced by lipid peroxidation [49]. The adverse effects are caused by oxidative stress, but can be minimized by the use of antioxidants [50]. It should be reported that doxorubicin induced a significant increase in apoptosis in

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S180 cells **(Table 1)**. However, this cytogenetic damage was also modulated by AA in 70% **(Table 2)**.

There are reports on the effect of antioxidants such as ascorbic acid, tocopherol and carotenoid that can inhibit the effects of various cytostatic drugs (5-fluorouracil, doxorubicin and vincristine) through cultures and cell lines [51, 52], which may reduce toxic effects in cellular systems [53].

There are many patients who are undergoing cancer treatments using micronutrient supplements to minimize side effects of the therapies. There are many matches on the effect of antioxidants that can decrease the effects of chemotherapy, but also mounting evidence of the antioxidant benefits at high concentrations, as pro-oxidants for the cytotoxic effects. In this context, it is important to explore the use of antioxidants and other additional micronutrients as an educational strategy on the potential beneficial and negative effects [54].

It was previously reported that ascorbic acid modulates apoptosis induction by anticancer CPA, DOX and AC, however, by adding apoptosis, the data show that antineoplastic also induce necrosis, which has been modulated by the AA 30% to 40% (Table 2). Chemotherapy is usually well tolerated, nevertheless, results of treatments that induce necrosis are predictive of the risk for metastasize, and, this information can be useful for systemic therapies [55].

Chemotherapy-induced apoptosis involves activation of the mitochondrial death pathway, which is tightly regulated by the balance between pro- and anti-apoptotic of B-cell lymphoma 2 (Bcl-2) family proteins. The activation of Bcl-2 pro-apoptotic proteins lead to permeability of mitochondria and release to the cytosol apoptogenic factors, such as, cytochrome c. The cytochrome c participates in the activation of caspase-9, which, in turn, activates effector caspases [56].

Different paths to cell death have been observed, including apoptosis recognized by nuclear fragmentation, with activation of caspases and absence of inflammation; autophagy characterized by the presence of large vacuoles which induces an inflammatory response; and necrosis that are not controlled, characterized by membrane disruption and induction of inflammatory responses [57].

In the cytogenetic damage by apoptosis and necrosis, as observed in photomicrography profile in Figure 2, CPA, DOX and AC also induced micronuclei. However, ascorbic acid modulated only CPA (70%) and AC (42%). Yet, other cytogenetic damages like nucleoplasmic bridges were observed, but they have not been modulated by AA **(Table 1)**. The micronuclei are formed from a whole chromosome or a fragment of one, and they are induced by genotoxic stresses, so they are closely related to carcinogenesis [58].

Conclusion

Firstly, this study indicates that the antineoplastic CPA, DOX and AC induce apoptosis, necrosis and other cytogenetic damages like micronuclei, nucleoplasmic bridges and nuclear buds in S180 cell line. Secondly, ascorbic acid can interfere with the apoptosis induction mechanisms and genetic aspects that are also clinically point to the effectiveness of cancer therapy.

Acknowledgements

Conflict of Interest

The authors declared no conflict of interests.

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