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Depolarization of mitochondrial membrane and generation of reactive oxygen species: mechanism of Sorbitol induced apoptotic cell death in human tumoral and non-tumoral cells

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Abstract

Literature on the cytotoxicity of sorbitol is limited and contradictory. In this study, organelle-specific cytotoxicity of 10-70 mg/mL of sorbitol using Alamar blue (for mitochondrial activity) and Neutral red (for lysosomal activity) in the human epithelial non-tumor cell, HEK 293, and tumor cell, MCF 7, was investigated. A comparative evaluation of the potential anti-tumor effect of sorbitol and doxorubicin, a known breast cancer drug, on MCF 7 cell line was also carried out. The present study investigated the alterations in mitochondrial membrane potential (MMP), cell cycle analysis (CCA), and reactive oxygen species (ROS) generation as the key molecular mechanisms underlining the induced cytotoxicity. Also, ethidium bromide and acridine orange double staining was used for further confirmation of apoptosis/necrosis. The data obtained revealed that sorbitol is cytotoxic to non-tumor cells, and the selectivity index showed that sorbitol is selectively cytotoxic to tumor cells, a chemotherapeutic potential not sufficiently explored so far. A synergistic probable anti-tumor effect of sorbitol in combination with doxorubicin was found. Apoptosis caused by disturbances in the membrane potential of the mitochondria and reactive oxygen species were the probable sorbitol mechanisms of action. Further studies are required so as to safeguard the public health against indiscriminate consumption of sorbitol, and to further elucidate its potential as an anticancer agent.

Introduction

In the last decade, there has been an increased demand for sugar alternatives in the drug and food industries. This is a result of increased market demand for sugar-free products as a result of their perceived health benefits over products containing sugar. Higher sugar consumption of sugar or sugar-containing products is associated with many health problems such as obesity, dental caries, and cardiovascular diseases, due to a rapid increase in the levels of blood sugar causing adverse effects on the consumers (Howard & Wylie-Rosett, 2002; Findikli & Turkoglu, 2014).
There has been a long interest by food manufacturers and consumers in dietary sweeteners which can serve as replacements for sucrose in foods by enhancing food flavor but with reduced calories, however, their safety has been controversial. Sugar substitutes have less food energy even though they duplicate sugar effect like taste as food additives. They can be synthetic or natural. Among the sugar substitutes are sugar alcohols also called polyols. They are generally less sweeter than sucrose, possessing similar properties and potential application in preparation of diverse food products.

Sorbitol, which is also known as glucitol, is one of the sugar alcohols naturally found in fruits such as apples, pears, and cherries. Sorbitol is a vitally important raw material extensively used in the light, chemicals, food, and pharmaceutical industry. It is approximately 60% as sweet as sucrose (Walters, 2009), water-soluble, thermosetting, and has been used in diverse dietary products including soft drinks, jams, candies, biscuits, cereal bars, and chocolates. Apart from being a food industry sweetener, sorbitol is also a chelant, antifreeze agent, thickener, vehicle, plasticizer, humectant, and emulsifier. Furthermore, it is capable of improving the aftertaste of other sweeteners like saccharin (Cardoso et al., 2016). Sorbitol has been reported to have therapeutic abilities as a result of its diuresis dehydration property. It has been used for the treatment of glaucoma, cerebral edema, and edematous oliguria of normal cardiac function. Study has also shown sorbitol to possess antitumor capability (Lu et al., 2014).

In recent time, the consumption and application of sorbitol have increased rapidly because it is considered a natural material, since it's a general believe that natural materials are safer when compared with their artificial counterparts. However, being a natural additive does not guarantee its safety. Despite the wide usage of sorbitol in diverse consumer products, the cytogenotoxicity study of sorbitol has been limited. The report of Johannes et al. (1992) in Chinese hamster ovary cells showed significant induction of DNA fragmentation after exposure to sorbitol. Similarly, Aquilano et al. (2007) reported cell death through apoptosis in human erythroleukaemia cells treated with sorbitol. Also, human peripheral lymphocytes had significant DNA damage after exposure to sorbitol using comet assay as reported by Findikli & Turkoglu (2014). Recently, Alabi et al. (2019) showed that oral consumption of sorbitol for 30 consecutive days by mice induced a dose-dependent significant increase in abnormal sperm morphology and bone marrow micronuclei. The report also showed a significant alteration in the oxidative parameters of the treated mice.

Consistent ingestion of food sweeteners can induce cytotoxic, genotoxic, and carcinogenic effects in the exposed individuals, hence, there is a need for further studies on the potential cytotoxicity of sorbitol so as to reach a scientific conclusion on its safety for human consumption. Therefore, this study aimed at evaluating the organelle-specific cytotoxic potential of sorbitol in (1) human epithelial (HEK 293) cell line, so as to elucidate the effect of sorbitol on normal cells, and (2) human breast tumor (MCF 7) cell line, to elucidate the anti-tumor potential for possible cancer-drug formulations. Alterations in MMP, CCA, and

ROS generation were studied as the molecular mechanisms underlining the induced cytotoxicity and apoptosis.

Materials and Methods

Materials

Sorbitol was purchased from Dinamica Quimica Contemporanea LTDA, Brazil, and Dimethyl sulfoxide from Merck (Darmstadt, German). GIBCO (Grand Island, NY, EUA) supplied the fetal bovine serum (FBS), antibiotics (streptomycin/penicillin), and media for cell culture, while Sigma-Aldrich (St. Louis, MO, USA) supplied 2′,7′-dichlorofluorescein diacetate (DCFH-DA), 5,5′,6′6-tetrachloro-,1′,3,3′ tetraethylbenzymidazolcarbocianyne iodide (JC-1 probe), and all other reagents.

Cell culture

We acquired the HEK 293 (human kidney epithelial cells) and MCF 7 (human epithelial breast tumor cells) from the cell bank of the Federal University of Rio de Janeiro, Brazil. The cells were cultured in DMEM and fortified with FBS (heat-inactivated, 10%), 100 μgmL^{-1} streptomycin, 10 mM HEPES, 100 UmL^{-1} penicillin and maintained at pH 7.4, 5% CO_2 humidified atmosphere, and 37 °C was used to culture the cells. The cells were passaged after every 2-3 days, by the addition of fresh medium after the removal of 90% of the initial supernatant. Before starting of each experiment, the cell viability is ensured by performing Trypan Blue exclusion assay.

Organelle-specific cytotoxicity test

Mitochondrial activity: Alamar blue test

Assessment of the cytotoxicity of sorbitol was carried out using Alamar Blue (resazurin) assay by following the protocol outlined by Ahmed et al. (1994) with slight modification. Briefly, 10000 cells/well were seeded in a 96-well plate for a period of 24 hours. The cells were exposed to 10 µL/mL of the final concentrations of 70.0, 52.5, 35.0, 20.0 and 10.0 mg/mL (highest concentration lower than 50 g/day acceptable dietary daily intake for 72 kg human) (FDA, 2015) of sorbitol for a period of 24 hours. Alamar blue medium (0.1 mg/mL) was used to replace the medium after incubation and re-incubated for 2 hours at 37 ^{o}C, after which the plates were put on the shaker for 30 seconds. The absorbance was measured in the spectrofluorimeter (Perkin Elmer LS 55, Boston, MA, USA). Calculated cell viability represents percentage of the control while the optical density of cells without sorbitol (control group) was taken as equivalent of 100% viable cells. The CC_{50} (concentration needed for reducing the number of cells by 50%) of sorbitol was determined using the Prism 5.0 software. Each assay was repeated thrice and performed in triplicate.

Lysosomal activity: Neutral red (NR) uptake test

The method of Borenfreund & Puerner (1984) was used for the Neutral red uptake test with slight modification. The cells were exposed to different concentrations of sorbitol same using Alamar blue test for 24 hours, after which we remove the medium and introduced a a fresh medium with neutral red solution (0.4% NR in water) into each well. Following incubation that lasted 2 h, cell washing was done using phosphate buffer and 1% acetic acid solution in ethanol was used to dissolve the cell monolayer. At room temperature, plates were shaken for

10 min for solubilization of dye, followed by careful aspiration of the supernatant into a 96-well plate for measurements. A microplate ELISA reader (EFOS 9305, Russia) was used to measure the optical density at a wavelength of 540 nm. In a similar way to Alamar blue test, the viability calculations of the results of this assay were also carried out.

Comparative anti-tumor effect of sorbitol with doxorubicin on MCF 7

To compare the potential anti-tumor effect of sorbitol, a 96-well plate was used to seed 1×10^4 cells/well of MCF 7. After 24 hours, cells were exposed to 10 µL/mL of 2.0, 4.0, and 6.0 µg/mL of doxorubicin; or the CC_{50} of sorbitol; or 1:1 mixture of 2.0 and 4.0 µg/mL of doxorubicin and 10.0, 20.0, 30.0 and 35.0 mg/mL of sorbitol for 24 hours. Doxorubicin is a known anti-cancer drug regularly prescribed to treat breast cancer. Neutral red test was used for the viability test after 24 h as described above.

Cell cycle analysis (CCA)

The flow cytometry described by Yang et al. (2007) was used for the analysis of cell cycle in exposed cells. Briefly, a 12-well plate was seeded with 4×10^5 cells/well for 24 hour and then exposed to different concentrations of sorbitol ($\leq CC_{50}$) before re-incubation for extra 24 hours. The cells were then centrifuged at room temperature for 10 minutes at 400 × g. After that cells were washed with PBS, and re-centrifuged at the same initial conditions. We removed the supernatant and fixed the cells with 70% ethanol at 4°C for 30 minutes. Subsequently, a solution of BSA (2%) with PBS was added before centrifugation for 10 min at 400 × g. The supernatants were then decanted and cells were permeabilized with 100 µg/mL RNase and lysis buffer (Triton-X 0.1% in PBS). FACS Canto flow cytometry equipment (Becton Dickinson, San Jose, CA, EUA) was used for analyzing the DNA content of the cells stained with propidium iodide (20 µg/mL) and software was used for determining the cell population in each phase of the cell cycle.

Mitochondrial membrane potential (MMP)

The disrupting ability of sorbitol on the membrane potential of the mitochondria of treated cells was studied with JC-1, the lipophilic cationic fluorescent probe, following the method proposed by Cossarizza et al. (1993) and later on modified by Alabi et al. (2013). Briefly, 12-well plates were seeded for 24 hours with 5×10^5 cells/well before exposure to sorbitol (same as in cell cycle analysis) and were subsequently incubated at 37ºC for 24 h. Thereafter, the supernatants containing sorbitol were decanted and JC-1 (10 µg/mL) was added to the cells, which was then incubated for 20 min at 37 ºC. A Nikon Eclipse TS100 inverted fluorescence microscope was then used for the visual assessment of the cells. The cells were harvested by trypsinization, rinsed twice with PBS before and subsequently suspended in PBS for fluorescence measurement using a Perkin-Elmer LS55 spectrofluorimeter. JC-1 was excited at 485nm, and the green and red emission fluorescence was detected at 535nm and 590nm, respectively (Cossarizza et al., 1993). For each sample, the red/green fluorescence was computed and normalized to represent 100% untreated control. A reduction in this fluorescence ratio was used as an indication of alterations in mitochondrial potential. A chemical electron transport uncoupler, CCCP (carboxyl cyanide *m*-chlorophenyl hydrazone) (1 µM), served as the positive control.

JC-1 was excited at 485 nm, with emissions detected at 535 nm for green fluorescence and 590 nm for red fluorescence (Cossarizza et al., 1993). The red-to-green fluorescence ratio was calculated for each sample and normalized to represent 100% of the untreated control. A reduction in this fluorescence ratio indicated changes in mitochondrial potential. Carboxyl

cyanide m-chlorophenyl hydrazone (CCCP) (1 μM), a chemical that uncouples electron transport, was used as the positive control.

Reactive oxygen species (ROS) generation

Following the method adapted by Alabi et al. (2013),the evaluation of intracellular reactive species was carried out using DCFH-DA (20,70-dichlorofluorescein diacetate). following Plates (12-well) were seeded for 24 hours with 15000 cells/well and then treated with the varying concentrations of sorbitol (same as in cell cycle analysis) and incubated at 37ºC for 24 h. Following incubation and removal of supernatant containing sorbitol, we incubated the cells in the dark with DCFH-DA (10 μM) for 30 minutes at 37ºC. Thereafter, we harvested the cells and washed 4 times with PBS. The quantification of green fluorescence used for the evaluation was carried out at wavelengths of 480nm for excitation and 520nm for emission (Sauer et al., 2003). The fluorescence readings were presented as the mean ± SEM of ROS levels, Fluorescence readings normalized to total protein content were expressed as mean ± SEM of ROS were compared with the untreated control and normalized to total protein content. Hydrogen peroxide (2 mM) served as the positive control.

Morphological Identification for Cell Death

3,6-dimethylaminoacridine (acridine orange) and 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide (ethidium bromide) double staining were used for the identification of necrosis and/or apoptosis in sorbitol-treated cells. Briefly, 4×10^5 cells/well seeded in 12-well plates were incubated with sorbitol (same as in cell cycle analysis) for 24 h. Following the incubation, the cells were washed with PBS twice after removing the medium, then stained with the mixture of acridine orange/ethidium bromide (1:2.25). Fluorescence microscopic imaging (Eclipse TS100, Nikon, Melville, NY, EUA) was used for the visualization. Image J software using a cell counter plugin (National Institute of Health, Bethesda, MD, USA) was used to analyze the images obtained at 200 × magnification for identification of cell death type and evaluation of morphological alterations. Accordingly, (a) viable cells appeared to have a green nucleus with intact structure; (b) cells with a bright-green nucleus and chromatin condensation (early apoptosis), in addition to dense orange areas of chromatin condensation (late apoptosis) were considered apoptosis; and (c) cells presenting orange intact nucleus depicted secondary necrosis.

Statistical Analysis

Each result was expressed as means ± standard error of the mean (SEM) of triplicates from a minimum of three independent experiments. ANOVA was used to analyze differences between the control and the different concentrations, and also Dunnett's test. A significance at $p<0.05$ was used.

Selectivity index (SI)

The ratio of the CC_{50} of sorbitol between non-tumor cells (HEK 293) and cancer cells (MCF 7) corresponds to the selectivity index (SI). When the SI is higher than one, this is an indication that the substance selects cancer cells more than normal cells (Winter et al., 2014). The methodology for the calculation of the CC_{50} of sorbitol in both cell lines after incubation for 24 h has been described above.

Sorbitol and Doxorubicin Interaction Analysis

To examine the interaction between sorbitol and doxorubicin, the isobologram analysis was carried out using the software CompuSyn 1.0 version. Synergy, additive, and antagonism effects occur when the interaction index is less than 1.0, equal to 1, and greater than 1, respectively (Foucquier & Guedi, 2015).

Results

Organelle-specific cytotoxicity

In order to ascertain if induced cytotoxicity of sorbitol in HEK 293 and MCF 7 cell lines was specific to a specific cellular organelle or general, cell viability was assessed through two tests: Alamar blue which detects mitochondrial activity majorly, and neutral red which detects lysosomal function. The results of cytotoxicity of sorbitol using Alamar blue in HEK 293 and MCF 7 are shown in Figure 1A. The data showed concentration-dependent cytotoxicity of sorbitol with a CC_{50} of 55.0 and 35.0 mg/mL for HEK 293 and MCF 7, respectively (Table 1). Figure 1B shows the cytotoxicity of different concentrations of sorbitol in HEK 293 and MCF 7 cells using the Neutral red test. Values of CC_{50} of 52.5 and 35.0 mg/mL of sorbitol were obtained for HEK 293 and MCF 7 cells, respectively (Table 1). In HEK 293, sorbitol appears to alter the lysosomal function of the cell more than mitochondrial activity. However, in MCF 7 cells, there was no organelle-specific preference between lysosomal function and mitochondrial activity. A selectivity index of 1.57 suggests that sorbitol is selective to MCF 7 cells in relation to the non-tumor HEK 293 cell line (Table 1). The comparative study of sorbitol with doxorubicin on the MCF 7 cell line using the neutral red test is shown in Figure 1C. The data showed that the CC_{50} of sorbitol (35.0 mg/mL) is as active as 4.0 µg/mL of doxorubicin. However, the use of 35.0 mg/mL of sorbitol concomitantly with 2.0 µg/mL of doxorubicin was more effective than 6.0 µg/mL of doxorubicin alone. A synergistic anti-tumor effect of sorbitol in combination with doxorubicin was observed at 30.0 and 35.0 mg/mL sorbitol + 2 µg/mL doxorubicin; and 10.0, 20.0, 30.0 and 35.0 mg/mL sorbitol + 4 µg/mL doxorubicin (Figure 2).

Table 1: CC_{50} of sorbitol in HEK 293 and MCF 7 cells using Alamar blue (AB) and Neutral red (NR) assays, and selectivity index (SI) of tumor and non-tumoral cells

Cell line	CC_{50} (mg/mL) AB	CC_{50} (mg/mL) NR	SI*
HEK 293	55.0	52.5	1.57
MCF 7	35.0	35.0	

* SI corresponds to the ratio of the CC_{50} of sorbitol between HEK 293 and MCF 7.

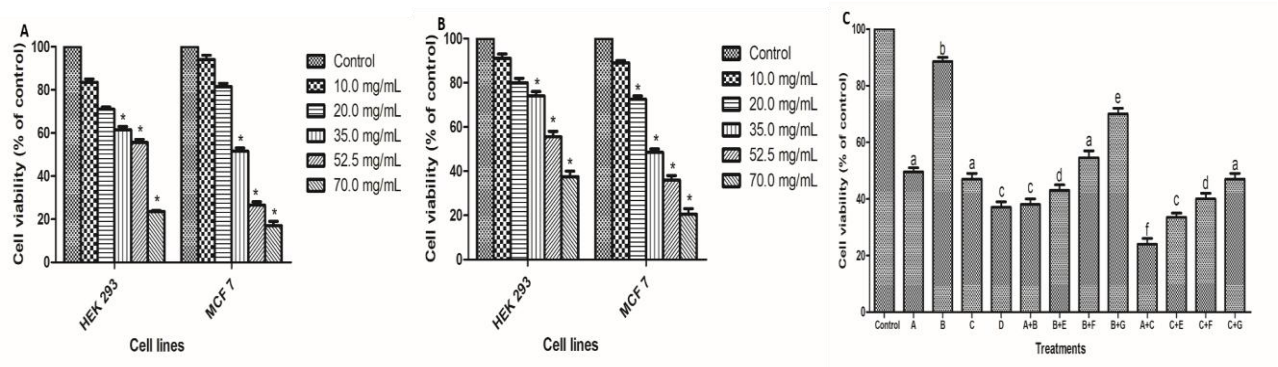

Fig. 1 Toxicity of sorbitol to MCF 7 and HEK 293 cell lines (A) Alamar blue and (B) Neutral red assays (C) Comparative toxicity of sorbitol and doxorubicin to MCF 7 cell line. The results of three experiments are represents as the mean ± SEM and optical density is taken as 100% of cell viability. The symbols * and alphabets a, c, d, e, and f reveal that results are statistically significant ($p<0.05$) compared to the control. Different alphabets show a significant difference while the same alphabet is not significantly different. A = 35.0 mg/mL of sorbitol, B = 2.0 µg/mL of doxorubicin, C = 4.0 µg/mL of doxorubicin, D = 6.0 µg/mL of doxorubicin, E = 30.0 mg/mL of sorbitol, F = 20.0 mg/mL of sorbitol, G = 10.0 mg/mL of sorbitol.

Cell cycle analysis

The incubation of MCF 7 and HEK 293 cells with different concentrations of sorbitol induced DNA fragmentation in both cell lines (Figure 3 A and B), as evidenced by the cell cycle assay analysis. Cells at the subdiploid (sub/G_1) phase significantly increased ($p<0.05$) corresponding to cell death by apoptosis in both HEK 293 and MCF 7. A significantly increased percentage of sub/G_1 phase cells from 4% in the control HEK 293 cells to 12% in 35.0 mg/mL sorbitol-incubated cells (a three-fold increase compared to the control), and 4% in the control MCF 7 to 15% in 35.0 mg/mL sorbitol- incubated cells (~ two-fold increase in comparison with the control) was observed. Also, there was a significant reduction ($p<0.05$) in G_2/M phase cells from 19% in control to 4% in sorbitol treated MCF 7 cells. Figure 1 of supplementary material presents the histograms of the cell cycle phases in HEK 293 and MCF 7 cells.

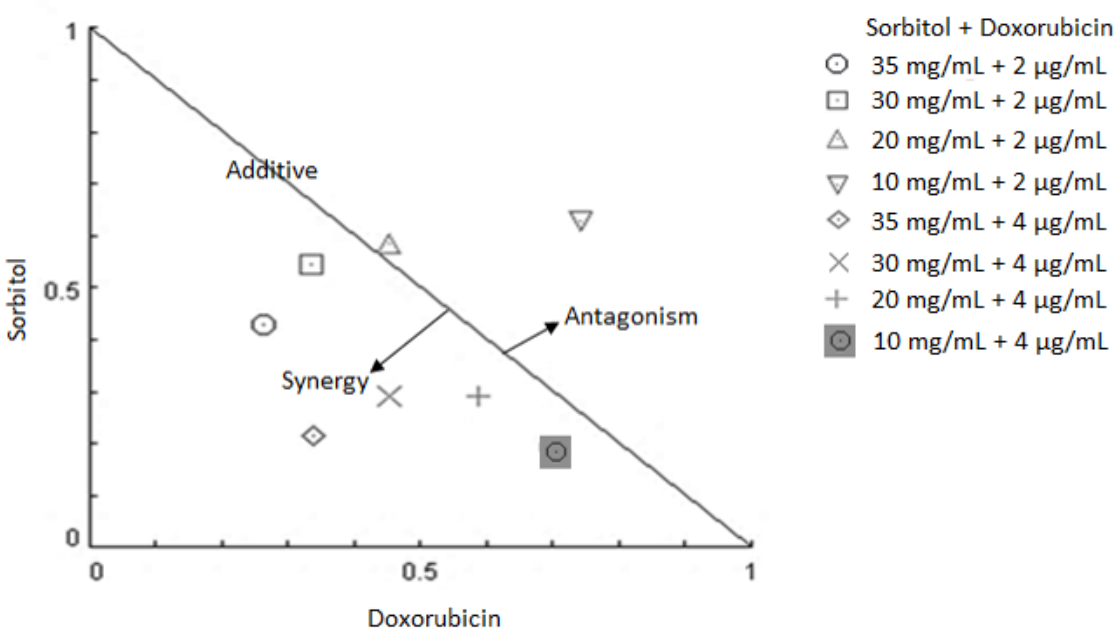

Fig. 2 Normalized Isobologram for drug combination. A normalized isobologram analysis depicting the interaction between different concentrations of sorbitol and doxorubicin in inhibiting the MCF 7 cell line growth. S*olid line*, concentrations of the combination that cause a 50% inhibition of cell proliferation (CC_{50}). The software CompuSyn 1.0 was used for the computation of the combination index.

Mitochondrial membrane potential (MMP)

Figure 3C shows the data obtained from the assessment of MMP of MCF 7 and HEK 293 cells which were treated with different concentrations of sorbitol. A concentration-dependent, significant decrease ($p<0.05$) in the MCF 7 and HEK 293 MMP in response to the incubation with different concentrations of sorbitol was recorded. This was evident with the diminution of the red to green fluorescence ratio intensity. In comparison to HEK 293 cells, sorbitol induced the decrease of MMP more significantly in MCF 7 cells at all the tested concentrations.

Reactive oxygen species (ROS) generation

The ROS generated in MCF 7 and HEK 293 cells after incubation with different concentrations of sorbitol is represented in Figure 3D. The results indicated that sorbitol triggered an increase in ROS generation in both cell lines but at a lower concentration in HEK 293. A significant increase in the generation of ROS was recorded at 20.0 and 35.0 mg/mL of sorbitol in HEK 293 but only at 35.0 mg/mL of sorbitol in the MCF 7 cell line.

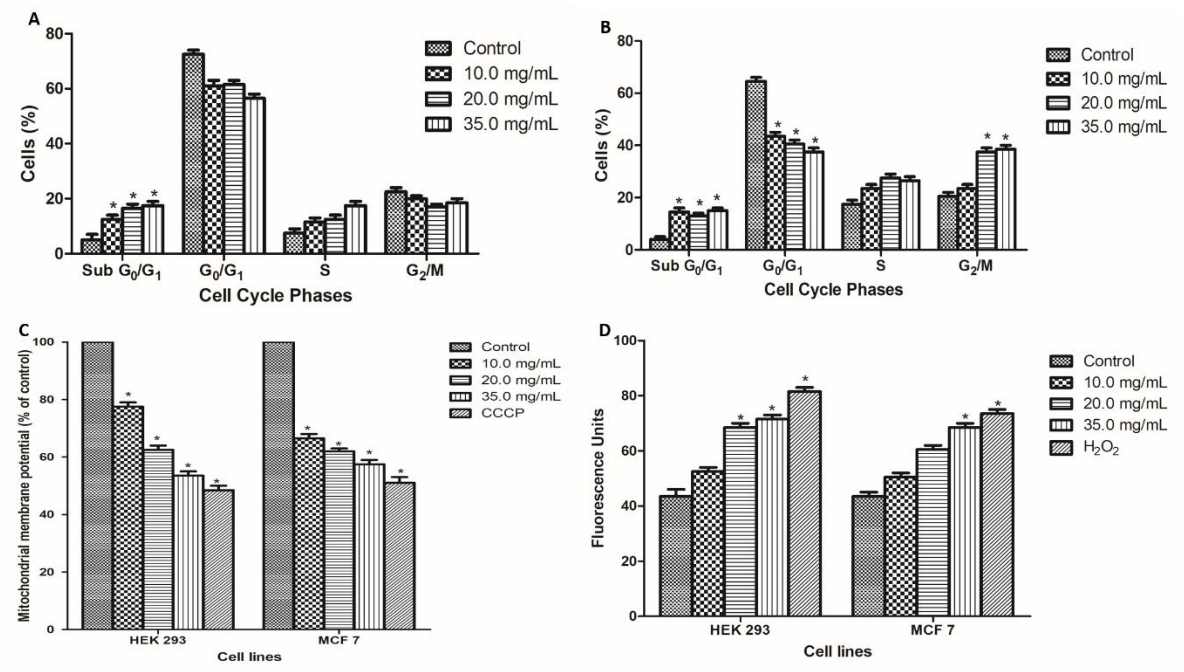

Fig. 3 The top panel portray effect of different soributol concentrations on (A) MCF 7 and (B) HEK 293 cell cycle distribution assessed after 24 hours exposure. Cells were stained with PI and analysis was carried out with flow cytometry. Flowing 2.5.1. software is used for analyzing the cellular DNA profile, with data represented as the percentage of number of cells in each cycle phase. The bottom panel represents mean ± SE of three experiments expressed as percentages (C) MMP of HEK 293 and MCF 7 and (D) ROS generation in HEK 293 and MCF 7 cell lines. *The results are considered significant at p-value < 0.05 compared to control.

Morphological Identification for Cell Death

As shown in Figure 4, sorbitol caused DNA fragmentation in HEK 293 and MCF 7 cells, an indicative of late apoptosis after incubation by varying sorbitol concentrations of sorbitol, as shown by fluorescence microscopic analysis. In the HEK 293 cells exposed to sorbitol, there was a record of chromatin condensation and nuclear fragmentation which are morphological

features of apoptosis. However, there were alterations in the shape and size of the sorbitol-exposed MCF 7 cells also, especially after incubation with 35.0 mg/mL of sorbitol in addition to morphological features shown by HEK 293 cells. No necrotic cells were recorded in both HEK 293 and MCF 7 and cells after incubation with sorbitol.

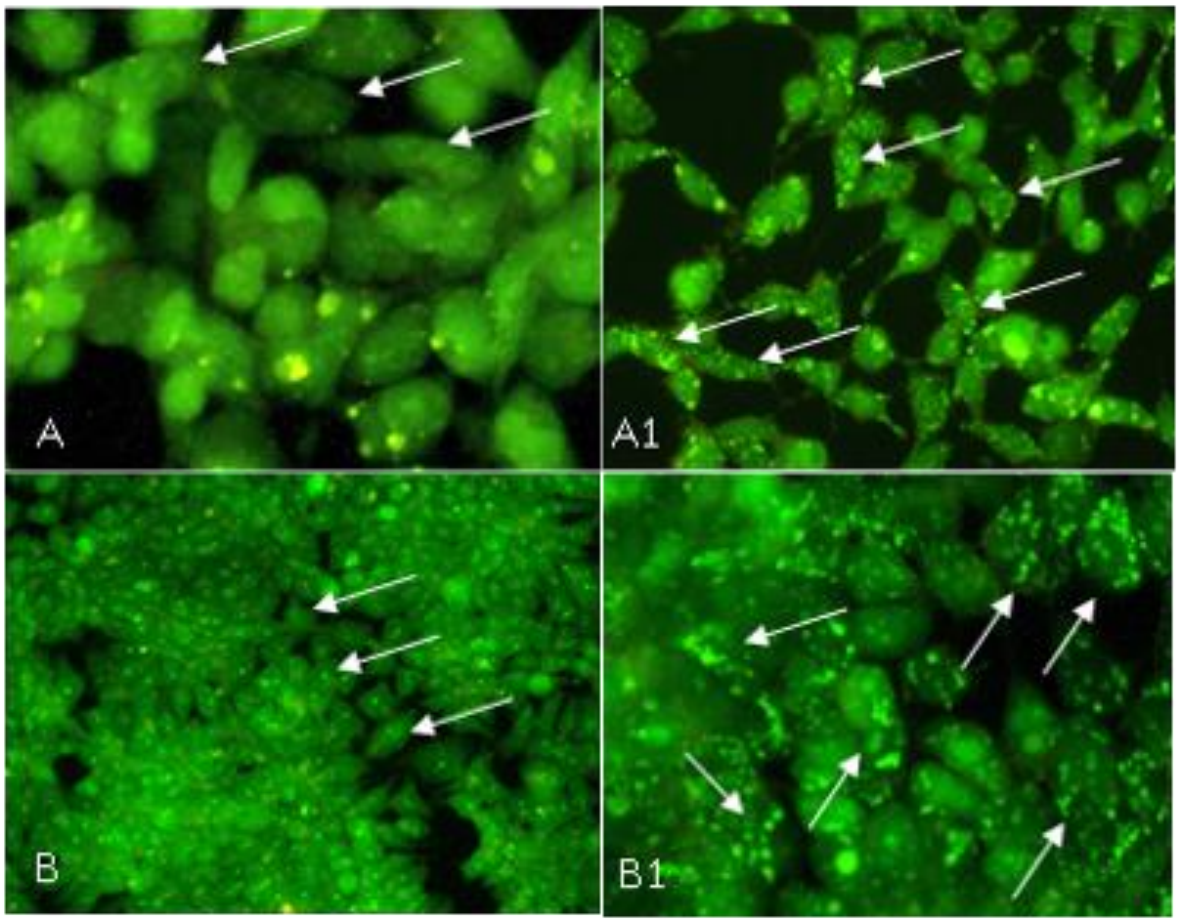

Fig. 4 The top panel (A) HEK 293 control cell (A1) HEK 293 cell line incubated with 35.0 mg/mL sorbitol showing apoptotic cells in representative images qualitatively analyzed using fluorescence microscope at a magnification 400 times. The bottom panel shows the alternation in morphology and apoptotic cells (B) MCF 7 control cell (B1) MCF 7 cell line incubated with sorbitol 35.0 mg/mL.

Discussion

Sorbitol is one of the sugar alcohols that is widely used in the drug and food industries as a sweetener. In this study, we evaluated organelle-specific cytotoxicity of sorbitol in human epithelial cells for the potential effect on exposed individuals and the potential anti-tumor property of the molecule. Also, we studied the molecular mechanism for induced cytotoxicity via cell cycle analysis, apoptotic pathway induced by ROS, and depolarization of the mitochondrial membrane.

In this study, our data showed that sorbitol is cytotoxic to both tumoral and non-tumoral cells with a CC_{50} of 52.5 and 35.0 mg/mL for HEK 293 and MCF 7 cell lines, respectively. Sorbitol cytotoxicity for non-tumoral cells is an indication of the potential danger of sorbitol consumption at high concentrations and the possible effect of its persistent long-term use on

biological systems. A CC_{50} of 52.5 mg/mL of sorbitol further indicates that the 50 g/per/72kg daily allowable intake in humans (FDA 2015) could have a negative health effect. At present, sorbitol is used in horseradish sauce, chewing gum, jams, and sweets at 110, 400, 80-100, and 350-950 mg/mL, respectively (Food Intolerance, 2017). Although the joint European Union's Scientific Committee and the FAO/WHO Expert Committee on Food Additives (JECFA) have classified some of these sweeteners as safe for consumption, the data in the present study indicate that sorbitol might be cytotoxic. In addition, Findikli & Turkoglu (2014) in their report have shown the potential genotoxicity of sorbitol, while Johannes et al. (1992) showed that sorbitol is capable of damaging the DNA of Chinese hamster ovary cells. Cardoso et al. (2016) also documented that exposure to sorbitol during lactation induced genotoxicity and metabolic alteration in the exposed rat's offspring. There is therefore a need for more studies on the safety of human consumption of sorbitol.

Conversely, the data of this study also show that sorbitol has potential anti-tumoral activity. Human breast tumoral cell line, MCF 7, incubated with sorbitol showed 50% of cell death at a concentration lower than the CC_{50} for non-tumoral cells, an indication that sorbitol is more cytotoxic to tumoral cells than to non-tumoral cells. Furthermore we confirm the selective cytotoxicity by selectivity index of greater than 1 (SI= 1.57). The selective cytotoxicity of sorbitol to tumor cell is moderate; indicating its potential as an anti-cancer drug. Lu et al. (2014) suggested that sorbitol could be a potential chemotherapeutic agent for cancer treatment. For assessing Sorbitol's anti-tumor efficacy, the CC_{50} was compared with varying concentrations of doxorubicin, a well-known anti-cancer drug used for the treatment of breast cancer. The findings reveal that 35.0 mg/mL of sorbitol comparable activity to 4.0 µg/mL of doxorubicin, and a combination of 35.0 mg/mL sorbitol with 2.0 µg/mL doxorubicin showed greater toxicity than 6.0 µg/mL of doxorubicin alone, indicating the synergic effect of the combination. Our results underscore the potential anti-tumor properties of sorbitol either as used as a standalone drug or in combination with existing drugs in breast cancer treatment.

The results further indicated that in non-tumor cells, sorbitol was found to cause cytotoxic effects by changing lysosomal activities, rather than affecting mitochondrial function. This observation suggests that the lysosome is more susceptible to sorbitol than the mitochondria. However, in the tumor cells, there was no organelle-specific cytotoxicity, an indication that sorbitol equally alters both mitochondrial and lysosomal activities of the exposed cells. The reported cytotoxicity of sorbitol in the present study is in agreement with literature where sorbitol has been documented to induce cytotoxicity in human erythroleukemia cells K562 (Aquilano et al., 2007), human colorectal cancer cells (Lu et al., 2014) and cytogenotoxic to rat bone marrow cells (Cardoso et al., 2016) and human lymphocytes (Findikli & Turkoglu, 2014). Canimoglu & Rencuzogullari (2013) also reported the cytogenotoxic effect of maltitol, a similar sweetener, in human peripheral lymphocytes.

To elucidate the molecular mechanism of cytotoxicity induced by sorbitol, we carried out a cell cycle analysis of sorbitol-exposed cells using flow cytometry and propidium iodide dye. The data showed that sorbitol induced DNA fragmentation, as indicated by a significant increase in subdiploid (sub/G1) phase cells. Cells in the subdiploid phase are considered apoptotic, which are set for cell death due to DNA fragmentation (Ormerod, 2002). The presence of apoptotic cells was further confirmed by ethidium bromide and acridine orange double staining. The data showed that the observed cell death type induced by sorbitol in MCF 7 and HEK 293 cells was apoptosis, not necrosis, as no necrotic cells were found. This conclusion is supported by the study of Lu et al. (2014), where sorbitol induced apoptosis in cancer cells of human colorectal.

To further elucidate the sorbitol-induced apoptotic pathway, we evaluated the generation of ROS and a possible disturbance in the mitochondrial membrane potential of cells treated with sorbitol. Obtained results showed a significant increase in the generation of ROS in both the non-tumoral and tumoral cell lines. The data also showed an increased generation of ROS at a lower concentration in non-tumoral cells compared to the tumoral cells. ROS are by-products of mitochondria metabolic reactions of aerobic cells and it occurs as a response to different stimuli, however, they can also be generated due to endoplasmic reticulum stress, which can make the inner membrane of the mitochondrion to be depolarized (Malhotra & Kaufman, 2007, Alabi et al., 2020). Hence, increment in ROS has been regarded as either the downstream factor or causative of the impairment of mitochondrial function and integrity, which is typical of the process of apoptosis (Simon et al., 2000; Aquilano et al., 2007). ROS has been shown to participate in both early and late stages of apoptosis (Ferrira et al., 2013), and according to Dreher & Junod (1996), high or moderate concentrations of ROS can become cytotoxic, thereby hindering cells proliferation and lead to cell death by apoptosis. Therefore, the cytotoxicity of sorbitol through apoptosis in this study is believed to be caused by the increased ROS generation with no corresponding increase in antioxidant defenses. The ability of sorbitol to generate ROS in treated cells reported in this study is consistent with the findings of Aquilano et al. (2007).

The increase in ROS generation has been linked to mitochondrial membrane permeability, causing respiratory chain damage and prompting the process of apoptosis (Valko et al., 2006, Alabi et al., 2020). For verifying ROS-induced mitochondrial membrane permeability in cells treated with sorbitol, we use JC-1 probe to monitor the MMP. The results showed a significant decrease in MMP after treatment with sorbitol in both tumoral and non-tumoral cells. Also, the disruption observed in the MMP was more pronounced in tumoral cells than in non-tumoral cells, suggesting that mitochondrial membrane disruption could be a major mechanism for sorbitol-induced cell death in tumoral cells. Thus, sorbitol appears to be an agent capable of compromising mitochondrial integrity, thereby activating the tumor cell death pathways and preventing tumor cell proliferation. The study demonstrated that sorbitol induces apoptosis through ROS generation and disruptions in the energy potential of the cell, leading to cell death.

The cytotoxicity induced by sorbitol in non-tumoral cells suggests a possible cytotoxic effect in exposed subjects. Also, we reported cytotoxicity of sorbitol in tumoral cells, an indication of the potential of sorbitol as a chemotherapeutic agent for cancer treatment. The induced cell death by sorbitol in both non-tumoral and tumoral cells was determined to be by apoptotic pathway. We suggest that the molecular mechanisms underlying the apoptotic pathway are due to the generation increase in ROS and disruption of MMP of the acquiesced cells. This study calls for further investigation into the potential cytogenotoxic effects of sorbitol, so as to safeguard public health against indiscriminate use and consumption of sorbitol, and to identify its potential use for cancer treatment.

Statements and Declarations

Competing interest

The authors declare that there are no conflicts of interest related to this study.

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