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Evaluating The Cytoprotective and Genoprotective Effects of Vitamin C Against Doxorubicin on normal human skin melanocytes

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Abstract

Chemotherapy is one of various cancer therapeutic treatments that usually has efficacy in cancer treatment, but it has many toxic or side effects on other normal tissues or cells. The present study involves the reduction of some toxic effects, such as cytotoxicity and DNA damage in normal human skin melanocytes (HFB4) that was exposed to doxorubicin (Dox) as a chemotherapeutic drug. HFB4 cells were treated by Dox alone and Dox which was combined with vitamin C (Vit. C). The MTT technique, alkaline comet assay as well as biochemical parameters were used. The results showed that Dox treatment alone at 0.26 and 0.39 µg/ml caused oxidative stress and DNA damage as well as caused the reduction of superoxide dismutase activity and the increase of lipid peroxidation. However, addition of Vit. C at concentrations of 32, 65, and 130 µg/ml reduced these effects on HFB4 cells. Also, it increased the proliferation of HFB4 cells and decreased the DNA breaks. Further, the addition of Vit. C with Dox reduced the lipid peroxidation and enhanced the anticancer activity of Dox. Conclusion: The combination of natural compounds (Vit. C) at low concentrations with Dox may be able to protect normal cells from genotoxcity and may be enhanced the anticancer activity of Dox against cancer cells.

Keywords: Vitamin C; Cytoprotective; 8-OHdG; Alkaline comet assay

1. Introduction

Chemotherapy is one of several different cure methods for the dangerous, fatal, and silent disease**;** cancer. For instance, chemotherapy drugs involving cisplatin, carpoplatin, and oxaliplatin have been used for about three decades in the treatment of several kinds of cancers like testicular, ovarian, and cervical [1]. Many or almost all chemotherapy drugs involving platinum drugs cause a lot of harmful side effects to normal tissues. Cisplatin raises creatinine levels in kidney cells as well as levels of tumour necrosis factor alpha (TNF-α), interlukin-6 (IL-6), interlukin-8 (IL-8) and interlukin-1 (IL-1), indicating the toxicity of cisplatin in the kidney [2].

Doxorubicin (Dox) is another anticancer drug that has been used for more than 30 years, but it has toxic effects on many vital organs [3]. It has high efficiency against breast cancer cells (MCF-7 cells and MDA-MB) and decreases the growth of cancerous cells. It also induces apoptosis via raising the expression of pro-apoptotic genes like Bcl-2-associated X protein (BAX), caspase-8, and caspase-3 and reducing the expression of anti-apoptotic gene like B-cell lymphoma 2 gene (Bcl-2). Additionally, it reduces the viability of non-tumorigenic epithelial cells as a normal cells (MCF-10F) that leads to cell programmed death (apoptosis) [4]. Dox inhibits the growth of human lung carcinoma cells (A549), human cervix carcinoma cells (Hela) and human heptoma cells (Hep G2) at different concentrations [5]. It elevates oxidative stress in brain cells [6] and increases the level of malonaldehyde (MDA) in heart tissue, which indicates lipid peroxidation and heart damage [7].

The toxicity of these drugs may be reduced by using natural compounds with the anticancer drug

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as an attempt to minimise the side effects of these anticancer drugs, such as vitamin C (Vit. C). Vit. C reduces the toxicity of Dox via the reduction of ROS. Dox also raises the creatine kinase, p53, ASK-1, and P38 to a high level. Further, it increases the Bax/ Bcl-2 ratio and the reduction of cell viability. On the other hand, Vit. C that combined with Dox diminished the creatine kinase, p53, ASK-1, and p38 levels [8].

Vit. C has cytoprotective and genoprotective effects against various agents that affect the different normal cells like lymphocytes and bone marrow cells or tissues like the liver or lung [9-11]. Moreover, Vit. C has a wide protective effect against various drugs; it protects nervous cells from the neurotoxicity of tramadol. Also, it reduces the expression of pro-apoptotic p53 and anti-apoptotic Bcl-2 [12]. Furthermore, it diminishes the genotoxicity in testis and spleen tissues [13].

Also, Vit. C decreases the harmful effects on liver and kidney by reduction the of alanine transaminase (ALT), aspartate transaminase (AST) and creatinine levels almost to normal levels. These enzymes and compounds were resulted from injured liver and kidney via the treatment of albino rats by sodium nitrate + fast green + glycine mixture as food additives [14].

Vit.C also protects the cerebella in the brain from the toxic effects of methotrexate that affect the cortex layer of this part of the brain [15]. Also, it increases the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) levels [16]. Vit. C may protect liver tissue from the toxic side effects of methotrexate by acting as a scavenger of free radicals that produced during methotrexate treatment. The treatment of Vit. C before the methotrexate protects the liver cells by reducing the ALT, AST, and ALP levels. Likewise, the reduction of malonaldehyde (MDA) and the elevation of SOD levels as oxidative stress markers were noticed [17]. Vit.C has no toxic effects on kidney tissue and has a protective effect against ivermectin (Ive), which causes many toxic effects on the kidney by inducing free radicals formation in the kidney. The combination of Vit. C with Ive diminishes the creatinine in serum and the reduction of ROS level and matrix metalloproteinase-9 (MMP9) activity, in addition to the rise of CAT, SOD, and GPX levels [18].

The present study aims to evaluate the cytoprotective and genoprotective effects of Vit. C against Dox on normal human skin melanocyte cell line (HFB4). Also, the evalution of synergestic effect of vit. C on Dox to raise its anticancer activity against human epidermoid carcinoma cells.

2. Experimental

2.1. Materials

Folin ciocalteu phenol reagent was purchased from Sd Fine Chem Limited, India. The Superoxide dismutase kit and lipid peroxidation were purchased from Biodiagnostic, Egypt. While, 8-hydroxy2-deoxyguanosine kit was bought from R & D System, USA. Vitamin C was obtained from ALPHA CHEMIKA Company, Cairo, Egypt, and Doxorubicin was purchased from Cellcys, India.

Two cell lines were used: a normal cell line (Human skin melanocyte cell line (HFB4)) and a cancerous cell line (Epidermoid carcinoma cells (A431)). These cell lines were purchased from Vacsera, Giza, Egypt. Cells were seeded and were incubated in RPMI 1640 supplmented with L-glutamine and 10% fetal bovine serum in a CO_2 incubator at 37^{o}C and 5% humidity.

2.2. Methods

2.2.1. MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] method was used to determine the cell viability, IC_{50} (that is a concentration at which kill 50% of the cells) of tested compounds on HFB4 and A431 cell lines , the cytoprotective effect of Vit. C

against Dox on HFB4 cells and the enhancement of Dox against cancerous cells (A431).

The numbr of HFB4 cells that was required 1×10^4 cells per well of a 96-well plate were treated by Vit. C at concentrations from 300 to 1500 µg/ml (That were chosen to estimate cell viability to obtain the concentration which leads to kill 50% of the cells, IC_{50}) for 24 h at 37ºC and 5% CO_2. Also, the cells were treated by Dox at concentrations of 2, 5, 10, 15, 20, 25, 30, 35, and 40 µg/ml for 24 h. In the same manner, experiments were conducted with A431 cells at concentrations of 125, 250, 500, and 1000 µg/ml of Dox for 24 h. Then cells were washed with phosphate buffer saline pH 7.4 (PBS). 20µl of MTT solution were added to each well and the plate was shaken. The plate was incubated for four hours at 37ºC in darkness. The produced formazane was dissolved in 200 µl of dimethyl sulfoxide (DMSO), then the plate was shaken well and was incubated for one hour in the dark. The absorbance was measured by a micro-plate spectrophotometer (BioTek ELx800, Korea) at a wavelength of 570–630 nm [19]. The inhibition of 50% of viable cells (IC_{50}) was calculated by using Graph Pad Prism version 6.

To investigate the cytoprotective impact of Vit C against Dox, the cell lines were treated by Vit. C at 32 µg/ml (this concentration represents the 5% of IC_{50} of Vit. C against HFB4 cells) before (pre-treatment) and after (post-treatment) the Dox treatment at IC_{50} in addition to the co-treatment.

2.2.2. *In vitro* alkaline comet assay

The HFB4 cells were treated by Vit. C at concentrations of 32, 65, and 130 µg/ml (5, 10 and 20% of IC_{50} of Vit. C) and the Dox treatment at concentrations of 0.26 and 0.39 µg/ml (10 and 15% of IC_{50} of Dox against HFB4 cells) for 24 h. In addition to the combination between the mentioned concentrations of Vit. C and Dox at concentrations of 0.26 µg/ml or 0.39 µg/ml. The cells were washed by PBS after incubation for 24 h, and they were suspended in 0.7% low melting agarose. The cell suspension was distributed on slides that were coated with normal agarose. The slides were allowed to solidify at 4ºC for 5 min, and then the slides were placed in lysis buffer at 4ºC for overnight. In the next step, the slides were placed in unwinding buffer, which is also called electrophoresis buffer, at 4ºC for 40 min. An electrophoresis step occurred in the unwinding buffer for 20 min at 25 V (1.0 V/cm). Then the slides were placed in 0.4 M Tris (pH=7.5). Finally, the slides were stained with ethidium bromide (20 µg/ml) and they were examined by using a fluorescent microscope which was connected via computer. The tail, head intensity (%), and tail moment were calculated by using the Comet assay IV software [20].

The comet assay was used to determine the biosafety of vitamin C on the DNA of the HFB4 cell line as well as the anti-genotoxicity of vitamin C on HFB4 cells that had been treated with Dox.

2.2.3. Biochemical analysis

2.2.3.1. Prepration of HFB4 cell lysate

The treated HFB4 cells suspension containing 4×10^5 cells was exposed to ultrasonic waves or impulses which were produced by an ultrasonic device (Human Lab) at 50/60 Hz for 90 sec [21]. The cell lysate was used for the measurement of MDA and SOD levels in addition to the determination of protien concentration.

2.2.3.2. Determination of malonaldehyde (MDA)

HFB4 cell lysate or standard (200 µl) was mixed with 1 ml of reaction mixture that contained thiobarbituric acid 0.025 M, detergent, and stabiliser (Biodiagnostic lipid peroxidation kit, Egypt). Then it was mixed well and boiled in a boiling water bath for 30 minutes. The mixture was cooled and the observed colour was measured at 534 nm [22].

2.2.3.3. Determination of superoxide dismutase activity (SOD)

For the determination of SOD, the HFB4 cells laysate was reacted with the reaction mixture (nitro blue tetrazolium 0.001 M, NADH 0.001 M, buffer solution pH = 8.5, 0.05 M), then phenazine methosulphate (PMS) $1x10^{-4}$ M was added to begin the reaction and measure the elevation of absorption at 560 nm for 5 min [23].

2.2.3.4. Determination of protein concentration

After protein precipitation of the HFB4 cells lysate, the precipitated protein was centrifuged and then dissolved in distilled water for protein determination.

1 ml of protein sample was added to 5 ml of the reagent that was comprised of 48 ml of 2% sodium carbonate in 0.1 N sodium hydroxide + 1ml of 1% sodium potassium tartrate + 1ml of 0.5% copper sulphate. The previous mixture was incubated for 10 min, then 0.5 ml of diluted Folin-Ciocalteau phenol was added and stirred well, and the sample was incubated in darkness for 30 min. After the incubation, the absorption of color was measured at 660 nm [24-25].

2.2.3.5. Extraction of DNA for determination of 8-OHdG

DNA was extracted from HFB4 cells using a DNA extraction kit from Jena Bioscience, Germany. The procedure of extraction occurred via cell membrane lysis, protein preciptation by adding protein preciptation solution of DNA extraction kit and centrifugation occurred at 15000 g for 1 min until the appearance of clear precipitated protein. Then the obtained supernatant was collected and isopropanol with a high purity of more than 99% was added to precipitate the DNA. Finally, centrifuging occurred at 15000 g for 1 min until a clear white pellet appeared, then the DNA was dissolved by adding the DNA hydration solution from the DNA extraction kit.

2.2.3.6. Determination of 8-Hydroxy-2-deoxy guanosine (8-OHdG)

The DNA extract from HFB4 cells or the dilutions of 8-OHdG were applied to 96 wells of an 8-OHdG-coated plate.The anti-8-OHdG monoclonal antibodies were then added and incubated at RT for one hour before being removed from the wells and washed four times with washing buffer (1X PBS + 0.1% Tween 20). The anti-mouse monoclonal antibody hourse reddish peroxidase (HRP) was added to wells and incubated for one hour at room temperature (RT). The solution was withdrawn from the wells and washed four times with washing buffer. Finally, the colorimetric substrate was added and incubated at room temperature (RT) for 15 min. before adding a stop solution of 0.2 M HCl (or 5% phosphoric acid) and measuring the absorbance at 450 nm [26].

2.2.4. Statistical Analysis

The data was displayed as mean±SD. Statistical analysis was conducted using one-way analysis of variance (ANOVA) and Duncan's multiple range test to obtain the difference between negative control and different treatments.

3. Results and Discussion

3.1 Cell viability and IC_{50} of Vit. C and Dox in HFB4 cells and A431 cells

The data were obtained from determination of the cells viability by using MTT method illustrated, Dox reduces the viability of HFB4 cells with the rise of concentration that expresses the cytotoxicity of Dox against normal cells, HFB4. The results are given in figures 1a and 1b. Also, the estimation of IC_{50} of Dox and Vit. C were obtained from prism graph programme by

using cell viability curve, that demonstrated the low value of IC_{50} of Dox (2.6 µg/ml) confirmed its cytotoxicity as shown in table 1. On the other hand, the IC_{50} of Vit. C (648.5 µg/ml) on HFB4 cells is relatively high, which indicates the biosafety of Vit. C. Moreover, figure 1c illustrated that Dox reduced the proliferation of cancerous skin cells (A431). It is noticed, that the high value of the IC_{50} of Dox against A431 cells (table 1) may be due to the low specificity of Dox against A431 cells.

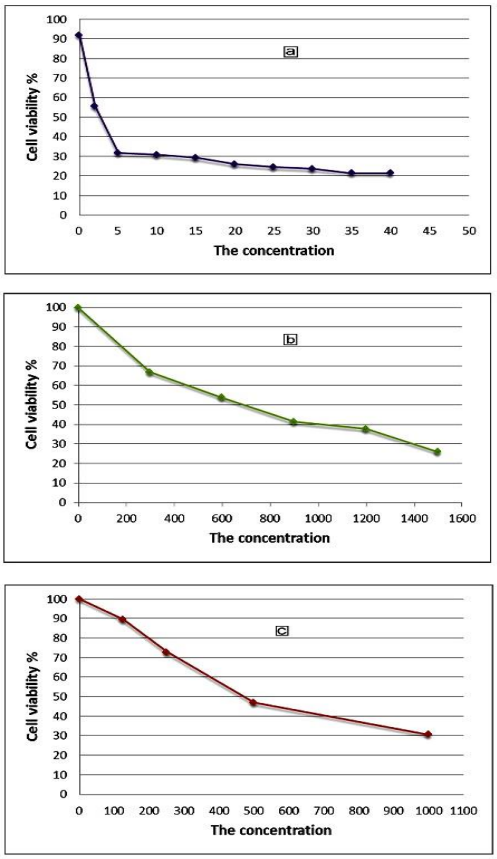

Figure 1. Cell viability curve of a) Dox on HFB4 cells, b) Vit. C on HFB4 cells, c) Dox on A431 cells.

Table 1. IC_{50} of tested compounds on two cell lines (HFB4 and A431)

Type of cell line	Tested compound	IC_{50} (µg/ml)
HFB4	Dox	2.6
HFB4	Vit. C	648.5
A431	Dox	470

3.2. The cytoprotective effect of Vit. C against Dox on HFB4 cells

The co-treatment, pre-treatment, and post-treatment of HFB4 cells by Vit. C at 32 µg/ml (That represents 5% of the IC_{50} on HFB4 cells) that were treated by Dox at a concentration of 2.6 µg/ml (that represents IC_{50} of Dox on HFB4 cells1). The results showed the significant increased of the cell viability of HFB4 cells that were treated by Dox at 2.6 µg/ml and they also pretreated and co-treated by Vit. C at 32 µg/ml (figure 2).

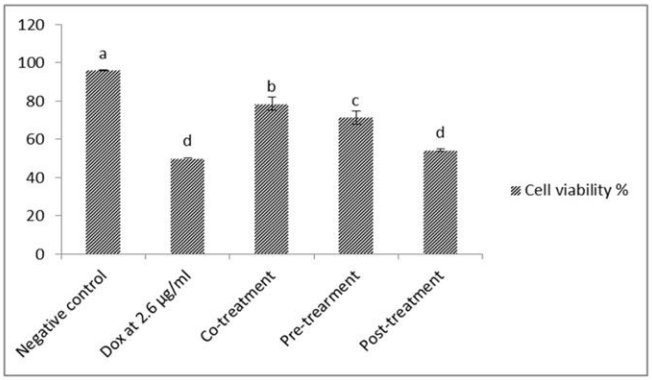

Figure 2. The cytoprotective effect of Vit. C at 32 µg/ml against cytotoxicity of Dox on HFB4 cells

Differences between control and treatment were conducted with Duncan's multiple range test after one-way ANOVA). The data in figure displayed the Mean±SD and the different small letters showed the different significance at $p<0.05$. Saline was used as a negative control.

These results revealed that the co-treatment and pre-treatment of HFB4 cells by Vit. C may reduce the cytotoxic effect of Dox by increasing the cell viability from 50% to about 70%. These data agreed with other studies, for instance, Dox caused cytotoxicity against cardiac myoblasts (H9c2) of rats as normal cells. The IC_{50} of Dox against H9c2 cells equals 0.177 µM and the pre-treatment of Vit. C elevates the concentration of IC_{50} of Dox on H9c2 cells by about three folds [27]. The present data can be confirmed with another study which revealed that Vit. C at 20 mM does not affect the normal cells [28]. While it reduces the number of abnormal

cells (cancerous cells) for different types of cancer cells to half the number (EC_{50}) at a concentration less than 4 mM. Further, Vit. C protects heart cells of mice that were injured by Dox by induction of apoptosis and reduction of cell viability [29]. The present data shows the anti-cytotoxicity or the cytoprotective effect of Vit. C against Dox at at 2.6 µg/ml on normal skin, HFB4 cells.

3.3. The enhancement of the therapeutic effect of Dox against cancerous cells (A431 cells)

The treatment of cells by Vit. C did not affect the anticancer activity of Dox on A431 cells. However, it improved the anti-cancer activity of Dox against A431 cells as cancrous cells that was noticed via the reduction of viability of A431 cells by more than 50% via the co-treatment, pre-treatment, and post-treatment of A431 cells by Vit. C at 32 µg/ml. The data is illustrated in figure 3.

The improvement of the anticancer activity of chemotherapeutic drugs against cancerous cells may be due to the anticancer activity of Vit. C. These results agreed with the study that showed vitamin C is able to diminish the proliferation of different breast cancer cells as cancerous cells [30, 31].

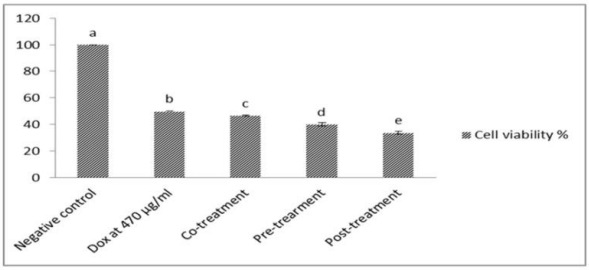

Figure 3. The enhancement of therapeutic effect of Dox by Vit. C at 32 µg/ml on A431cells. Differences between control and treatment were conducted with Duncan's multiple range test after one-way ANOVA). The data in figure displayed the mean±SD and the different small letters showed the different significance at $p<0.05$. Saline was used as a negative control.

3.4. The biosafety of Vit. C on DNA of HFB4 cells

Figure 4 shows that Vit. C at tested concentrations of 32, 65, and 130 µg/ml that concentrations represent 5%, 10% and 20% of IC_{50} of Vit. C on HFB4 cells, they did not cause significant ($p<0.05$) genotoxicity when they were compared to control (cells were treated by saline).

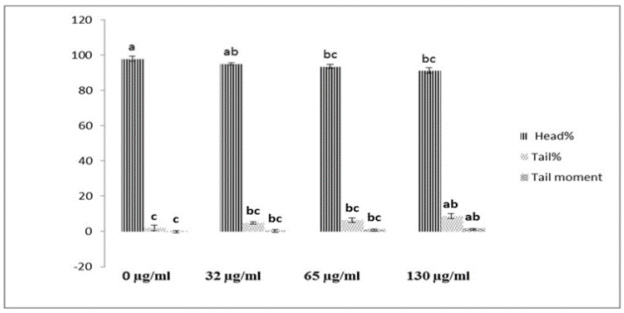

Figure 4. Comet assay results of DNA of HFB4 cells treated by Vit. C at 32, 65, and 130 µg/ml. Differences between control and treatment were conducted with Duncan's multiple range test after one-way ANOVA). The data in figure displayed the mean±SD and the different small letters showed the different significance at $p<0.05$.

The comet data include three parameters head%, tail% and last parameter is a tail moment. The reduction of head% and the elevation of tail% and tail moment express the bad effect on DNA of tested cells that were treated by tested compounds or the genotoxicity of tested compound. The results showed that the head% of Vit. C at concentrations of 32, 65, and 130 µg/ml were 95, 93.6, and 91.3%, respectively, that means they have no significant influence when they were compared with the head% of untreated cells (97.9%) at $p<0.05$. Another comet parameter, tail% of cells that were treated by Vit. C at the same concentrations also have no significant effect at $p<0.05$, that was appeared via the reduction of tail% (5, 6.4, and 8.7 %, respectively) compared with the tail% of untreated cells. These results confirmed that non-genotoxicity of Vit. C at tested concentrations on the DNA of normal HFB4 cells . These findings agreed with the study that found Vit. C at 50

µg/ml has no genotoxicity on blood lymphocytes as normal cells [32].

3.5. The anti-genotoxicity of vitamin C on HFB4 by Dox treatment

Using an alkaline comet assay, Dox at concentrations of 0.26 and 0.39 µg/ml that represent 10% and 15% of the IC_{50} of Dox on HFB4 cells. They caused DNA damage which was appeared in figure 5 and 6 via the significant reduction of head% and inceasing of tail% and tail moment compared with untreated cells. The treatment of HFB4 cells by the combining Dox at 0.26 or 0.39 µg/ml with Vit. C at 32, 65, or 130 µg/ml diminished the genotoxic effects of Dox. This appears from the diminishing in the DNA damage parameters (Tail% and Tail momment) and the increase in Head %, as shown in figures 5–7. The tail% of DNA of cells treated by Dox alone at 0.26 and 0.39 µg/ml equals 11.48 and 17.58%, respectively. However, the co-treatment with Vit. C significantly (at $p<0.05$) diminished the tail % to 2–7% when the cells were treated by the Dox at 0.26 µg/ml combined with Vit. C at concentrations of 32, 65, or 130 µg/ml. While the combination of Dox at 0.39 g/ml and Vit. C at 32, 65, or 130 µg/ml reduced the tail from 17.58% to 6-9%. When Dox was combined with Vit. C at the tested concentrations, another parameter (the tail moment, which expresses DNA damage) decreased from 9.1 (Dox concentration = 0.26 µg/ml) to 0.3-3.2. The same effect happened when Dox at 0.39 µg/ml was combined with Vit. C at tested concentrations, the tail moment was diminished from 4.3 to 0.5-1.6.

On the other hand, Dox alone at 0.26 and 0.39 µg/ml has a head% equals 88.1 and 79.4%which significantly increases at $p<0.05$ when the cells were treated by the Dox at 0.26 µg/ml or 0.39 µg/ml with Vit. C at 32, 65, and 130 µg/ml. Consequently, the head% increased to 92-97% when the cells were treated by Dox at 0.26 g/ml and Vit. C at different tested concentrations, while the head% became 91-94% when the cells were treated by Dox at 0.39 µg/ml and different tested concentrations.

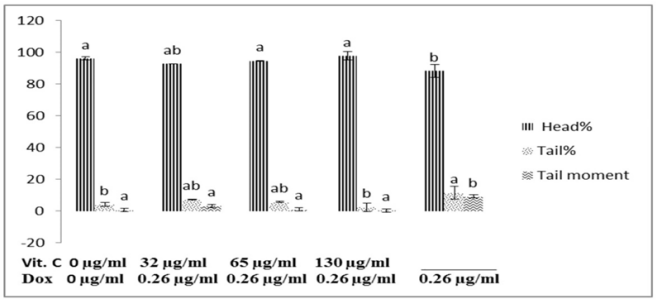

Figure 5. Illustration of comet assay of the genoprotective effect of Vit. C against the genotoxicity of Dox at 0.26 µg/ml. Differences between control and treatment were conducted with Duncan's multiple range test after one-way ANOVA). The data in figure displayed the mean±SD and the different small letters showed the different significance at $p<0.05$.

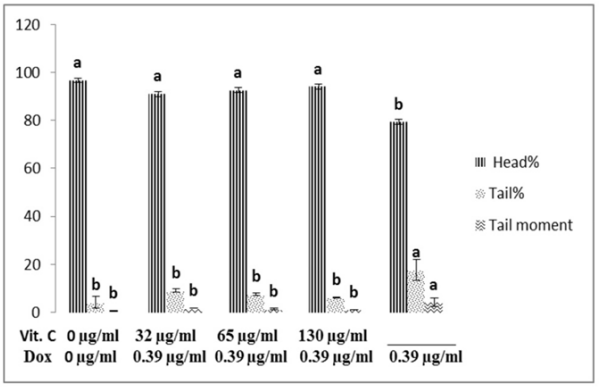

Figure 6. Illustration of comet assay of the genoprotective effect of Vit. C against the genotoxicity of Dox at 0.39 µg/ml. Differences between control and treatment were conducted with Duncan's multiple range test after one-way ANOVA). The data in figure displayed the mean±SD and the different small letters showed the different significance at $p<0.05$.

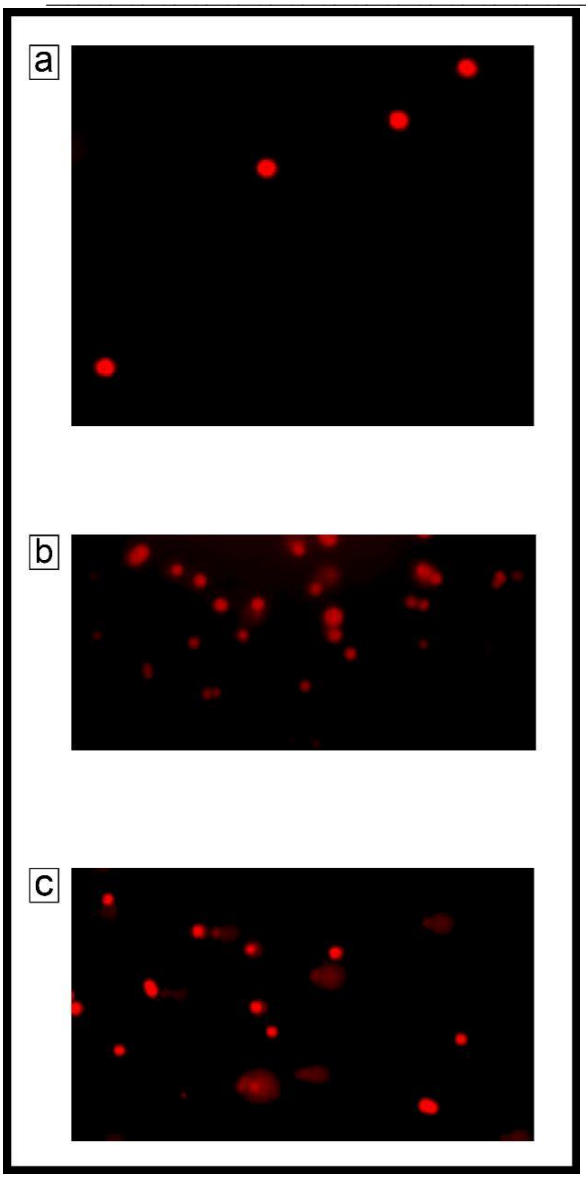

Figure 7. Comet assay from the treatment by Saline, Vit. C 32 µg/ml + Dox 0.39 µg/ml and Dox alone 0.39 µg/ml (a, b and c, respectively)

The results illustrated that Vit. C can protect normal cells against the toxicity of Dox on DNA. Another study demonstrated that Vit. C reduces the genotoxic effect of vincristine (VIC) and permethrin (PEN) in human blood lymphocytes via assessment of the genotoxicity of VIC and PEN by micronuclei assay. Vitamin C has a significant effect ($p<0.05$) in the reduction of micronuclei formation as a DNA damage indicator compared with the treatment by VIC or PEN [11].

3.6. Biochemical parameters

3.6.1. SOD activity and protein concentration of treated HFB4 cells

The treatment of cells by Dox reduced the SOD activity compared with untreated cells. However, the presence of Vit. C as a co-treatment with Dox reduced the SOD activity when it was compared with control and Dox, as shown in table 2.

Table 2. SOD activity of treated HFB4 cells by Dox at 0.39 µg/ml and Dox with different concentrations of Vit. C

No	Treatment	SOD activity (U/ml)
1	Negative control (untreated cells)	284.4±0.15[a]
2	0.39 µg/ml Dox+ 32 µg/ml Vit. C	73.13±0.21[c]
3	0.39 µg/ml Dox+ 65 µg/ml Vit. C	68.46±0.5[d]
4	0.39 µg/ml Dox+ 130 µg/ml Vit. C	64.65±0.01[e]
5	0.39 µg/ml Dox	112±0.05[b]

The data in the table displayed the mean ± SD and the different small letters showed the different significance at $p<0.05$.

The reduction in SOD activity when normal cells were treated by Dox may due to the oxidative stress which elevates ROS formation especialy superoxide radicals. This interpretation related to the study which showed that Dox caused cardiac mechanical impairments and increased ROS production, It also increased the activity of p38 MAP kinase [33, 34]. The low activity of SOD when Dox was combined with Vit. C could be attributed to vitami C's ability to reduce ROS formation.

The protein concentration decreased with the increasing of Dox concentration, but this

effect was diminished when Dox was combined with vit. C at different concentrations; as shown in table 3.

Table 3. Protein concentration of HFB4 cells lysate

Treatment	Protein concentration (µg/ml)
Negative control (untreated cells)	357.67±4.24[a]
0.26 µg\ml Dox+ 32 µg\ml Vit. C	300±9.43[e]
0.26 µg\ml Dox+ 65 µg\ml Vit. C	342±6.6[b]
0.26 µg\ml Dox+ 130 µg\ml Vit. C	307.335±3.77[de]
0.39 µg\ml Dox+ 32 µg\ml Vit. C	330.33±4.24[c]
0.39 µg\ml Dox+ 65 µg\ml Vit. C	318.665±3.77[d]
0.39 µg\ml Dox+ 130 µg\ml Vit. C	279±4.24[f]
0.26 µg\ml Dox	251.665±2.35[g]
0.39 µg\ml Dox	280±4.71[f]

The data in the table displayed the mean ± SD and the different small letters showed the different significance at $p<0.05$.

3.6.2. MDA concentration of treated HFB4 cells

Dox at 0.39 µg/ml caused lipid perioxidation via the presence of MDA and elevation of MDA levels in cell lysates compared with untreated cells. The combination of Dox with Vit. C at different concentrations (32, 65, and 130 µg/ml) reduced lipid oxidation and the effect is dependent tested concentration of Vit. C; the results are shown in table 4.

On previous studies, Vit. C diminished the oxidative stress that was caused by qunalphos. It decreased the MDA, TNF-α and IL-6 levels when rats were treated by qunalphos with Vit. C compared with positive control animals group. Further, Vit.C allivates the nephrotoxicity induced by vancomycin (VCN), which is an antibiotic [35, 36]. The data revealed that Vit.C decreases the oxidative stress and inflammation that was induced by VCN. On the other hand, the last study showed that Dox reduced the ferric ion to ferrous and it produced a Dox-ferrous complex. The Dox-ferrous complex forms different active radicals that can break the deoxyribose, which may form kinds of radicals that induce lipid peroxidation [37-38]. The MDA level in cells that were treated by Dox is about 8 nmol/ml , it was reduced to 2-4 nmol/ml via the treatment of the cells by Vit. C at different concentrations as shown in table 4. Hence, Vit. C reduced the oxidative stress by reducing the lipid peroxidation which was produced from Dox treatment, in turn decreasing the MDA level.

Table 4. MDA concentration in cells treated by Dox at 0.39µg/ml and Dox with different concentrations of Vit. C

Treatment	MDA concentration (nmol/ml)
Negative control (untreated cells)	4.105± 0.21[bc]
0.39 µg/ml Dox+ 32 µg/ml Vit. C	4.59±0.79[bc]
0.39 µg/ml Dox+ 65 µg/ml Vit. C	4.925±0.63[b]
0.39 µg/ml Dox+ 130 µg/ml Vit. C	2.945±0.47[c]
0.39 µg/ml Dox	8.175± 0.9[a]

The data in the table displayed the mean ± SD and the different small letters showed the different significance at $p<0.05$.

3.6.3. 8-OHdG concentration of DNA extract from treated HFB4 cells

Dox at concentration 0.39 µg/ml caused elevation of the production of 8-OHdG compared with DNA extract of untreated cells, this express the incidence of oxidative damage to DNA by Dox. On the other hand, vit. C reduced the 8-OHdG when the cells were treated by a combination of Dox with vit. C (results are shown in table 5). The increasing of 8-OHdG with increasing of vit. C that was combined with Dox may due to the induction of DNA repair by elemination of the abnormal nucleotides.

Table 5. 8-OHdG concentration in DNA extract from cells treated by Dox at 0.39 µg/ml and Dox with different concentrations of Vit. C

Treatment	8-OHdG concentration (ng/ml)
Negative control (untreated cells)	0.933 ± 0.05^{d}
0.39 µg/ml Dox+ 65 µg/ml Vit. C	1.278 ± 0.01^{c}
0.39 µg/ml Dox+ 130 µg/ml Vit. C	1.771 ± 0.01^{b}
0.39 µg/ml Dox	2.003 ± 0.04^{a}

The data in the table displayed the mean ± SD and the different small letters showed the different significance at $p<0.05$.

The current findings supported the study found that vitamin C reduced 8-OHdG levels in peripheral blood lymphocytes of chronic hemodialysis patients as well as ROS levels [39].

4. Conclusions

The present study can be concluded that the treatment of cells with Dox alone as an anticancer agent caused cytotoxic and genotoxic effects (*in vitro study*) using two techniques: MTT and comet assay) on both normal and cancer cells. However, the co-treatment with Vit. C as natural compound reduces the harmful effects of Dox as chemotherapeutic drug. Vitamin C treatment at low concentrations (32, 65 and 130 µg/ml) is able to protect normal cells (HFB4) against the cytotoxicity and genotoxicity of cancer chemotherapeutic drug (Dox), it also enhances the cytoxicity of Dox against cancer cells (A431).

5. Conflicts of interest

There is no conflict to declare.

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