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Kertas Asli/*Original Articles*

Combination Effect of Tamoxifen and Ascorbic Acid Treatment on Breast Cancer Cells (MCF-7) and Cervical Cancer Cells (HeLa)

Kesan Rawatan Kombinasi Tamoksifen dan Asid Askorbik ke atas Sel Kanser Payudara (MCF-7) dan Sel Kanser Serviks

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ABSTRACT

Breast cancer and cervical cancer are among the leading causes of death among women in the world. Even though chemotherapy is available as cancer treatment, the development of drug resistance in both cancer cells has reduced the efficacy of chemotherapeutic drugs in such treatment. The current study was aimed to evaluate the cell viability of human breast cancer cells, MCF-7, and cervical cancer cells, HeLa upon the combination treatment of ascorbic acid and tamoxifen. The cell viability was measured using the MTT assay, with an incubation period of 72 hours in a humidified CO_2 incubator. The concentrations of tamoxifen and ascorbic acid that reduced 50% of the cell population (IC_{50}) were determined from the dose-response curve. The IC_{50} concentration was used to determine the cell viability in the treated cells. CompuSyn software was used to evaluate the combined effects towards both cells upon treatment and the results were calculated as combination index (CI). The data were analyzed using GraphPad Prism (version 7). Statistical analysis was performed using an independent t-test. The IC_{50} values of tamoxifen and ascorbic acid on MCF-7 cells were 14.53 µg/ml and 15.8 µg/ml respectively, while the IC_{50} values of tamoxifen and ascorbic acid on HeLa cells were 11.09 µg/ml and 202.3 µg/ml respectively. The combination of tamoxifen and ascorbic acid exerted a greater growth reduction percentage in both cells compared to tamoxifen alone. The results indicated that ascorbic acid synergizes the cytotoxic effect of tamoxifen at lower concentrations towards MCF-7 cells with a CI less than 1. However, the combination of tamoxifen and ascorbic acid exerted an antagonistic effect in HeLa cells, with a CI more than 1.

Keywords: Combination treatment; ascorbic acid; tamoxifen; MCF-7 cells; HeLa cells; cell viability

ABSTRAK

Kanser payudara dan kanser servik adalah antara penyebab kematian dalam kalangan wanita di dunia. Walaupun kemoterapi tersedia sebagai rawatan barah, pembangunan kerintangan ubatan dalam kedua-dua jenis sel kanser tersebut telah mengurangkan keberkesanan rawatan ubatan kemoteraputik kanser. Kajian ini bertujuan untuk menilai viabiliti sel kanser payudara, MCF-7 dan sel kanser servik, HeLa yang dirawat dengan kombinasi asid askorbik dan tamoksifen. Pengukuran viabiliti sel dijalankan dengan asai MTT selepas dieram selama 72 jam di dalam inkubator CO_2 yang lembab. Kepekatan tamoksifen dan asid askorbik yang menurunkan populasi sel sebanyak 50% (IC_{50}) ditentukan daripada lengkuk dos tindak balas. Kepekatan IC_{50} digunakan untuk menentukan viabiliti sel yang terawat. Perisian CompuSyn digunakan untuk menilai kesan kombinasi terhadap kedua-dua jenis sel kanser terawat melalui pengiraan indeks kombinasi (CI). Data dianalisis menggunakan GraphPadPrism (Versi 7). Analisis statistik dilakukan dengan ujian-t tidak bersandar. Nilai IC_{50} tamoksifen dan asid askorbik ke atas viabiliti sel kanser MCF-7 adalah masing-masing 14.53 µg/ml dan15.8 µg/ml, manakala nilai IC_{50} tamoksifen dan asid askorbik bagi sel HeLa adalah masing-masing 11.09 µg/ml dan 202.3 µg/ml. Kombinasi tamoksifen dan asid askorbik menunjukkan peratusan perencatan pertumbuhan sel yang lebih tinggi berbanding tamoksifen bersendirian. Hasil menunjukkan asid askorbik mensinergi kesan sitotoksik tamoksifen pada kepekatan yang lebih rendah terhadap MCF-7 dengan nilai CI kurang daripada 1. Walau bagaimanapun, kombinasi tamoksifen dan asid askorbik menunjukkan kesan antagonis terhadap sel HeLa dengan nilai CI lebih daripada 1.

Kata kunci: Rawatan kombinasi; asid askorbik; tamoksifen; sel MCF-7; sel HeLa; viabiliti sel

INTRODUCTION

Cancer is one of the dreaded diseases in the world with millions of incidences of affected patients and associated deaths (Basu et al. 2017). Globally, breast cancer and cervical cancer are the most commonly diagnosed cancer in women and become the leading causes of cancer death in 110 and 36 countries, respectively (Sung et al. 2021). The 2020 statistics indicated that 6.9% and 3.4% of new death due to breast cancer and cervical cancer respectively (Sung et al. 2021)

Many drugs and chemopreventive agents targeting various pathways have been introduced to reduce cancer cell growth (Kaufmann & Gores 2000). Tamoxifen, which is a non-steroidal triphenylethylene derivate, has been used for years in chemotherapy and has successfully treated a variety of cancers (Bogush et al. 2012), including breast cancer (Davies et al. 2011). Although tamoxifen is effective in controlling breast cancer due to its antiestrogenic effects, it was found to have obvious carcinogenic effects on female reproductive organs. Studies discovered that tamoxifen causes estrogenic changes in the vaginal and cervical squamous epithelium and increases the incidence of cervical and endometrial polyps (Nasu et al. 2008). Also, it was reported to have a negative side effect of increasing the risk of endometrial cancer in postmenopausal women (van Leeuwen et al. 1994), associated particularly with rare tumour types (van Leeuwen et al. 1994; Bernstein et al. 1999) due to poor prognosis (Acharya et al. 2005). To date, there are no reports of studies or applications regarding the use of tamoxifen in the chemotherapy of cervical cancer (National Cancer Institute, USA 2020) mainly due to its characteristic as the selective estrogen receptor modulators (SERMs) associated with endometrial proliferation.

Tamoxifen was first reported to exhibit dose-dependent biphasic action in SFR cells, a subline of human cervical carcinoma, CaSKi. At low concentration (10^{-9} and 10^{-11} M), it stimulates cell growth whereas, at high concentration (10^{-6} and 10^{-7} M), it shows inhibitory effects (Hwang et al. 1992). However, the role of oestrogen in cervical cancer, which is strongly associated with human papillomavirus (HPV) infections, is still poorly understood (Chung et al. 2010).

Even though chemotherapy is the most effective cancer treatment to date (Fernando & Jones 2015), the development of drug resistance in cancer cells has reduced the efficacy of chemotherapeutic drugs. Regarding this, chemotherapy has been continuously improved over the years by combining other chemicals or drugs of natural or synthetic sources with known anticancer drugs to increase the efficacy of the treatment. In theory, combination drug therapy is more effective compared to single-drug therapy. However, it usually increases the toxicities (Lee & Nan 2012).

Antioxidant compounds have been studied extensively recently to enhance the efficacy of anticancer agents (Bayat Mokhtari et al. 2017). A cancer patient may be prescribed with certain supplements with antioxidant properties to assist the regeneration of new normal cells after chemotherapy (Greenlee et al. 2009). One of the supplements reported is ascorbic acid (Stephenson et al. 2013).

Ascorbic acid is a common natural antioxidant and has been long assumed to be essential for free radical clearance (Wang et al. 2017). However, studies have shown that high ascorbic acid concentrations can turn it into a prooxidant which disturbs the normal redox state of cells and cause a toxic effect through the production of reactive oxygen species, resulting in anticancer activity or inhibition of cancer cell growth (Rawal et al. 2013; An et al. 2013). Hence became the reason why ascorbic acid has been used in combination with other anticancer drugs in cancer treatment. Besides, its role in collagen production may also protect normal tissues from tumour invasiveness and metastasis (Mikirova et al. 2013). In addition, ascorbic acid replenishes the depleted of vitamin in cancer patients, which improves the immune system function and enhance the health and well-being of the patients.

Thus, in this study, ascorbic acid was postulated to have a role in modulating the estrogenic effect of tamoxifen in cervical cancer cells. On top of that, the combination of ascorbic acid and tamoxifen was expected to enhance the anticancer activity of the drug towards breast cancer cells. Both cells proliferation activity (IC_{50}) and the combination index (CI) were used as indicators in this study for forecasting the pathway of the underlying mechanism of the combination treatment, which is crucial for future works as tamoxifen alone exhibits multiple adverse effects in chemotherapy of breast cancer and developed cervical neoplasia in certain circumstances (Hwang et al. 1992). In this regard, this study aimed to determine the cell viability and CI of both the breast cancer cells (MCF-7) and cervical cancer cells (HeLa) treated with an anticancer drug (tamoxifen) and antioxidant compound (ascorbic acid).

METHODOLOGY

CELL CULTURE

Breast cancer cells (MCF-7) and cervical cancer cells (HeLa) were obtained from the stock culture deposited at the School of Health Sciences, Universiti Sains Malaysia. The cell line was grown under a temperature of 37°C in a

humidified incubator with 5 % CO_2. The mixture of Dulbecco's Modified Eagle's Medium (DMEM), 10 % of Fetal Bovine Serum (FBS) and 1 % of penicillin-streptomycin antibiotics was the complete medium used in culturing the cells. Cell culture work was conducted inside a Class II Biosafety Cabinet. The culturing steps were done with aseptic techniques and condition to prevent contamination.

CELL REVIVAL

The cryopreserved cell was thawed in a 37°C water bath for a while until the content inside the cryovial melted down. The content was transferred into a 15 ml Falcon tube and 1 ml of the complete medium was added to the tube before centrifuging the tube at 1500 rpm for 5 minutes at room temperature. While waiting for the centrifugation process, 4 ml of the complete medium was filled into the T25 cell culture flask until the surface of the flask was fully covered. Next, after the centrifugation process was done, the supernatant was removed and the cell pellet was suspended with 1 ml of the complete medium to avoid the cell from clumping. The cell suspension was then dispensed into the flask containing the complete medium and the cell morphology was observed under an inverted light microscope. The flask was labelled with the cell type, passage number and date. After that, the flask was placed in a 37°C CO_2 incubator.

TREATMENT OF CELLS

MCF-7 cells (4 x 10^3 cells per well) and HeLa cells (3 x 10^3 cells per well) were seeded in 96 well plates. The cells were incubated at 37°C. After 24 hours and the cells reached 80-90 % confluency, the medium was discarded. The cells were treated with 100 µL of ascorbic acid and tamoxifen at various concentrations. The treated cells were incubated in a humidified incubator with 5 % CO2 at 37°C for 72 hours. Treatments were carried out in triplicate.

DETERMINATION OF CELL VIABILITY AND IC_{50} WITH MTT ASSAY

MTT assay was performed in order to determine the IC_{50} of tamoxifen and ascorbic acid. First, 5 mg MTT powder was dissolved with 1 ml of phosphate buffer saline (PBS) and the solution was vortex mixed. Then, the mixture was filtered by using a sterile syringe filter into a 15 ml Falcon tube. MTT preparation must be done in a dark condition. After that, 10 µl of MTT solution (5 mg/ml in PBS) was added to each well. The 96-well plates were wrapped with aluminium foil and incubated in a humidified incubator with 5 % CO_2 at 37°C for 4 hours. After 4 hours of incubation, the solution in the wells was discarded. Following that, 100 µl of absolute DMSO was added into the wells to dissolve the blue formazan crystals formed by viable cells. The 96-well plates were then wrapped in aluminium foil and were put on a shaker for 5 minutes. Next, the absorbance (OD) of each well was measured using a microplate reader at wavelength 570 nm. The IC_{50} values of tamoxifen and ascorbic acid were determined from the dose-response curve by plotting a graph of the percentage of cell viability (%) against log10 concentration (µg/ml) using GraphPad Prism 6. The percentage of cell viability (%) was calculated by using the formula:

$$\text{Percentage of cell viability (\%)} = \frac{\text{Absorbance in a well}}{\text{Absorbance of negative control}} \times 100\ \%$$

The obtained IC_{50} values of tamoxifen and ascorbic acid were used in the combination treatment.

COMBINATION TREATMENT

For combination treatment, the experiment was done with a non-constant combination of tamoxifen and ascorbic acid concentrations. Generally, a non-constant combination is a random ratio combination, for example, drug A can be combined with drug B with five different combination ratios. Another feature of the non-constant combination is that the IC_{50} value of drug A (D1) can be kept at a fixed concentration while varying the IC_{50} value of drug B (D2), or vice versa (Chou & Martin 2005). The non-constant ratio combination design used in this study followed the procedure described by Wan Omar et al. (2016) with some modifications, in which serial concentrations of tamoxifen were combined with a fixed concentration of ascorbic acid.

MCF-7 cells were treated with tamoxifen at concentrations of 14.53 µg/ml, 7.27 µg/ml, 3.64 µg/ml, 1.82 µg/ml and 0.91 µg/ml in combination with ascorbic acid at a fixed IC_{50} concentration of 15.8 µg/ml. Meanwhile, HeLa cells were treated with tamoxifen at various concentrations (11.09 µg/ml, 5.55 µg/ml, 2.77 µg/ml, 1.39 µg/ml and 0.69 µg/ml) in combination with ascorbic acid at a fixed IC_{50} concentration of 202.3 µg/ml. The percentage of cell viability (%) was determined by MTT assay.

ANALYSIS OF SYNERGISTIC EFFECTS

The data of the combination treatment were analyzed using CompuSyn software to determine the synergistic, additive and antagonistic effects of the drugs combination. Statistical analysis was performed and the result was presented as a CI. The CI value is a mathematical and quantitative representation of the pharmacological

interplay of two drugs where CI>1 represents antagonism effect, CI=1 represents additive effect and CI<1 represent synergism effect (Qiao et al. 2015).

STATISTICAL ANALYSIS

The data were presented as means ± SD. The differences in the mean values between tamoxifen alone and the combination of tamoxifen and ascorbic acid were analyzed using GraphPad Prism (Version7) and the independent t-test. A p value less than 0.05 was considered statistically significant.

RESULTS

The cell viability of the cancer cells treated with tamoxifen and ascorbic acid was determined by MTT assay. Tamoxifen was observed to inhibit the growth of both types of cancer cells at a lower concentration, whereas ascorbic acid inhibited the growth of MCF-7 cells at a lower concentration while a higher concentration was required to inhibit the growth of HeLa cells. Figure 1 represents the dose-response curves of tamoxifen and ascorbic acid in MCF-7 cells, with the percentage of cell viability (%) plotted against log10 concentration (µg/ml). Based on Figures 1(A) and 1(B), the IC_{50} value of tamoxifen on MCF-7 cells was 14.53 µg/ml whereas the IC_{50} value of ascorbic acid on MCF-7 cells was 15.8 µg/ml. The results showed that the IC_{50} values of tamoxifen and ascorbic acid varied less than one-fold.

A non-constant combination treatment of tamoxifen and ascorbic acid on MCF-7 cells was designed to determine the combined effect of tamoxifen and ascorbic acid. The combination treatment was done with serial concentrations of tamoxifen combined with a fixed concentration of ascorbic acid at 15.8 µg/ml. The results showed that the combination of tamoxifen and ascorbic acid exerted greater MCF-7 cells inhibition at all concentrations compared to tamoxifen alone, as shown in Figure 2.

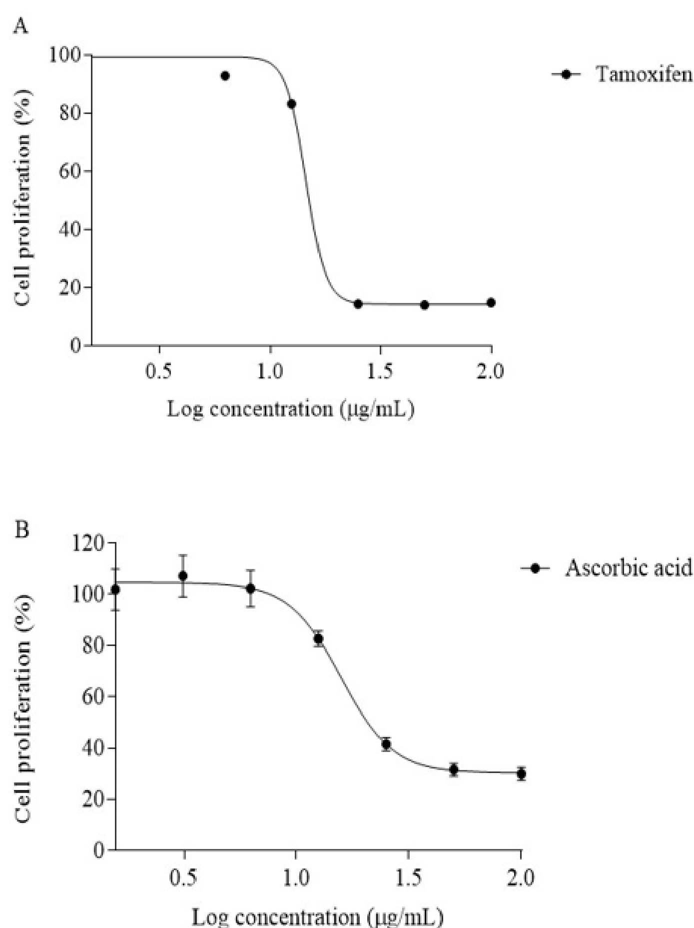

FIGURE 1. Antiproliferative activity of different concentrations of (A) tamoxifen and (B) ascorbic acid on MCF-7 cells. Each value represents the mean ± SD from three separate experiments

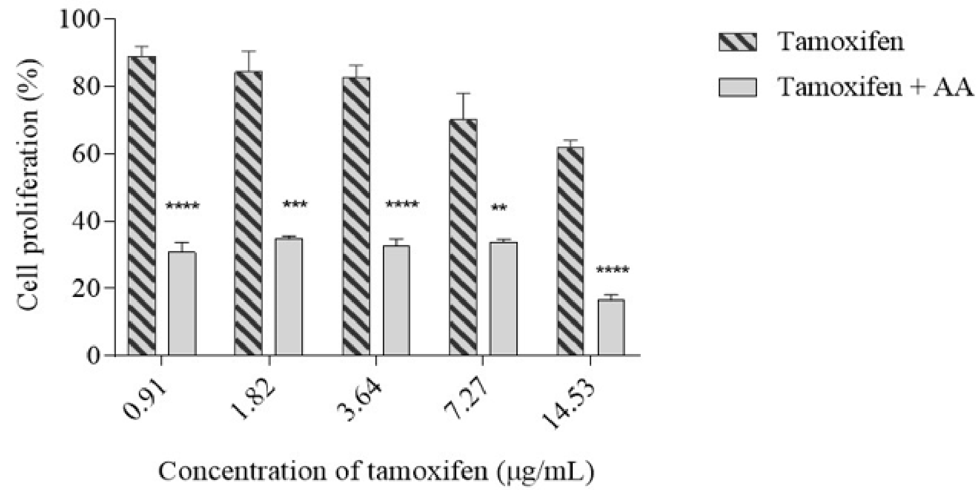

FIGURE 2. Antiproliferative effect of tamoxifen alone and tamoxifen in the combination with ascorbic acid (AA) on MCF-7 cells. Each value represents mean ± SD from three separate experiments. **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$

TABLE 1. CI values for the combined effect of tamoxifen and ascorbic acid on MCF-7 cells

Tamoxifen dose (µg/mL)	Ascorbic acid dose (µg/mL)	Effect	CI
14.53	15.8	0.8355	0.47823
7.27	15.8	0.6610	0.49734
3.64	15.8	0.6717	0.38308
1.82	15.8	0.6532	0.34245
0.91	15.8	0.6924	0.29255

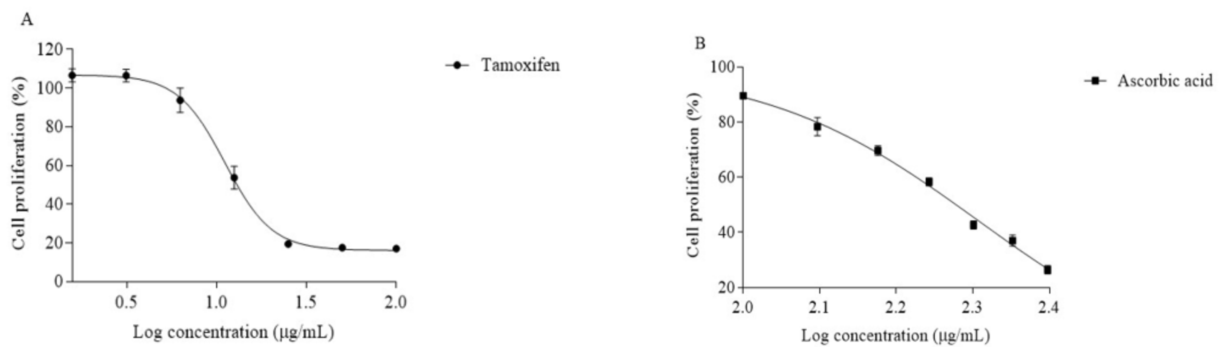

FIGURE 3. Antiproliferative activity of different concentrations of (A) tamoxifen and (B) ascorbic acid on HeLa cells. Each value represents the mean ± SD from three separate experiments

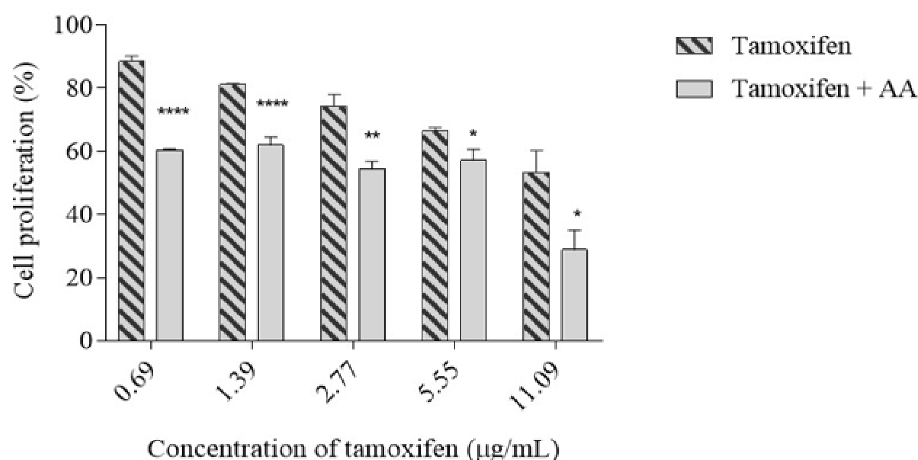

FIGURE 4. Antiproliferative effect of tamoxifen alone and tamoxifen in the combination with ascorbic acid (AA) on HeLa cells. Each value represents mean ± SD from three separate experiments. *: p<0.05, **: p<0.01, ****: p<0.0001

Table 2. CI values for the combined effect of tamoxifen and ascorbic acid on HeLa cells

Tamoxifen dose (μg/mL)	Ascorbic acid dose (μg/mL)	Effect	CI
11.09	202.3	0.7107	1.12028
5.55	202.3	0.4270	1.41649
2.77	202.3	0.4549	1.25020
1.39	202.3	0.3792	1.31009
0.69	202.3	0.3966	1.24904

All combinations of ascorbic acid (15.80 μg/ml) and tamoxifen (0.91 g/ml to 14.53 μg/ml) exhibited CI values less than 1, which indicated that the combination had a synergistic effect. The lowest CI value was observed in the combination of ascorbic acid and the lowest tamoxifen concentration (Table 1).

In this study, the antiproliferative effect of tamoxifen and ascorbic acid were also tested on HeLa cells. The IC_{50} values of tamoxifen and ascorbic acid obtained from the MTT assay were 11.09 μg/ml and 202.3 μg/ml, respectively. Tamoxifen, which is a wide spectrum chemotherapeutic drug, showed a higher cytotoxicity effect, with greater antiproliferative activity at a lower concentration (Figure 3). On the other hand, ascorbic acid required more than 10-fold higher concentration as compared to tamoxifen to inhibit 50 % of the cell population.

Like MCF-7 cells, a similar non-constant combination treatment of tamoxifen and ascorbic acid was designed to determine the combined effect of tamoxifen and ascorbic acid on HeLa cells. The combination treatment was done with serial concentrations of tamoxifen combined with a fixed concentration of ascorbic acid at 202.3 μg/ml. It was observed that the growth inhibition effect of the combination of tamoxifen and ascorbic acid (for all concentration combination) on HeLa cells was greater by 30 % to 70 % when compared to tamoxifen alone (Figure 4). All combinations had CI values of more than 1, which indicated an antagonistic effect (Table 2). In this study, the combination with a lower concentration of tamoxifen and the lowest CI value was chosen for further study.

DISCUSSION

The Selective Estrogen Receptor Modulators (SERMs), tamoxifen is the most commonly used drug for the treatment of breast cancer (Negreira et al. 2015). It is a potent antioxidant and a non-steroidal antioestrogen drug. Also, it is considered a prodrug since it is extensively metabolized in the body after administration, yielding several compounds with higher pharmacological activity than itself. These main active metabolites include 4-hydroxy-tamoxifen and 4-hydroxy-N-desmethyl-tamoxifen, also known as endoxifen (Reid et al. 2014). However, tamoxifen may damage both cancer and normal cells.

Although tamoxifen has its own anticarcinogenic potential, it produces some adverse toxic effects when taken for a long time (Muralikrishnan et al. 2010). The failure of tamoxifen in breast cancer treatment is because long term exposure to the drug may induce the emergence of tamoxifen-resistant ERα-positive cell clones, on which oestrogen may induce apoptosis and the emergence of a stem cell-like population of resistant cells (Notas et al. 2015). Tamoxifen resistance has been attributed to either a variability of tamoxifen metabolic fate by individual patients, concurring by the identification of low, normal and increased tamoxifen metabolites, or to the evolution of the disease phenotype during treatment with the emergence of tamoxifen-resistant clones (Notas et al. 2015). Thus, newer approaches that do not rely solely on the traditional cytotoxicity of a single agent are required in order to provide a more targeted, efficient and enhanced form of cancer therapy (Bayat Mokhtari et al. 2017).

One of the key approaches to overcome drug resistance is combination treatment (Kumar et al. 2016). Combination treatment would essentially increase the therapeutic index of cancer therapy and produce a more potent cytotoxic effect (Lee et al. 2019). Because cancer cells are heterogeneous, combination treatment can be alternated with standard therapy in order to eliminate all cancer cells (Bayat Mokhtari et al. 2017). In order to minimize the side effects of tamoxifen and improve its antioxidant efficacy in cancer treatment, a combination with other antioxidants is necessary (Muralikrishnan et al. 2010).

An antioxidant supplement can be broadly defined as any dietary supplement that exerts antioxidant actions. Antioxidant supplements taken during cancer treatment are believed to counteract the oxidative damages to healthy tissues, have a direct anticancer activity or confer general health benefits (Greenlee et al. 2009). One of the most commonly considered antioxidant supplements is ascorbic acid. The use of ascorbic acid in combination with cytotoxic chemotherapy is encouraged because it potentiates the antitumour activity (Espey et al. 2011; Stephenson et al. 2013). Not only that, Harris et al. (2014) reported that ascorbic acid supplementation reduced the total mortality and breast cancer-specific mortality rates among breast cancer patients.

Ascorbic acid has been occasionally used as a complementary treatment of cancer since 1974, to aid patients' survival and quality of life. Studies in humans, animals and *in vitro* showed that other antioxidants, such as tocopherols and carotenoids can also inhibit the growth of neoplastic cells, induce apoptosis, boost cell differentiation and inhibit the activity of protein kinase C and adenylyl cyclase, which considered as antitumour effects. Those findings also affirmed that a high-dose of ascorbic acid therapy can benefit patients by improving their prognosis and therapeutic efficacy (Gröber 2009). Even though ascorbic acid has been widely used in the treatment and prevention of cancer, nevertheless, the clinical findings are still inconclusive. It was observed that at low concentration, ascorbic acid exhibits an antioxidant role, preventing oxidation which induces apoptosis. However, a high concentration of ascorbic acid increases the production of adenosine triphosphate (generated by mitochondria), inducing apoptosis in tumour cell lines via a pro-oxidant mechanism (González et al. 2010).

Multiple studies had shown that ascorbic acid increases the efficacy of anticancer drugs. For instance, Guerriero et al. (2014) reported that when treating breast cancer cell lines with mitoxantrone and ascorbic acid, ascorbic acid increased the dose-dependent antineoplastic activity of mitoxantrone which influenced the apoptosis, cell cycle and cell signalling, hence, increased the cytotoxicity effect of mitoxantrone. Also, Catani et al. (2002) reported that ascorbic acid supplementation upregulated the MLH1 (MutL homologue-1) protein in human keratinocytes. On top of that, the cells supplemented with ascorbic acid showed greater sensitivity to apoptosis induced by cisplatin underlined by the activation of the MLH1/c-Abl/p73 signalling pathway (Catani et al. 2002). However, there are still many controversies regarding the role of ascorbic acid in the prevention and treatment of cancer.

In this study, it was found that tamoxifen had a cytotoxic effect on the MCF-7 and HeLa cells at lower IC_{50} value. This is because tamoxifen has a high affinity to oestrogen receptors, thus, enhancing its activity towards MCF-7 cells (Kelly et al. 2017). A large proportion of breast cancer types (including MCF-7) express oestrogen receptors and are dependent on oestrogens for their proliferation and survival (Negreira et al. 2015). The antiestrogenic effects of tamoxifen are responsible for its anticancer activities via competitive inhibition of oestrogen binding to oestrogen receptors, resulting in cellular apoptosis (Yaacob et al. 2013). Since HeLa cells are oestrogen receptor-negative, the action of the tamoxifen onto the cell was possibly by inducing the apoptosis and oxidative stress via the generation of reactive oxygen species (ROS) in the mitochondria, as reported by Rivera-Guevara et al. (2010).

In this study, the requirement of a higher concentration of ascorbic acid to inhibit the cell growth of HeLa cells might be due to insufficient concentration of ascorbic acid radicals and hydrogen peroxide to induce tumour cell death at lower concentration (Chen et al. 2007). A recent study reported that ascorbic acid at doses of 5 mM and 8 mM were required for a reduction in proliferation and viability of HeLa cell at 24 hours and 48 hours, respectively (Aastha Sindhwani et al. 2019). A high dose of ascorbic acid turns

it into a pro-oxidant therapeutic agent in cancer treatment by generating ascorbate radicals and hydrogen peroxide in extracellular fluid *in vivo* (Yeom et al. 2009). More recently, Unlu et al. (2016) also reported that a high dose of ascorbic acid can create a cytotoxic effect on cancer cells by means of chemical reactions that produce hydrogen peroxide. The higher IC_{50} value of ascorbic acid on HeLa cells indicated that ascorbic acid becomes pro-oxidant at a higher concentration by increasing adenosine triphosphate production that induces apoptosis of cancer cell line (Mata et al. 2016).

Also, the results showed that the effect of ascorbic acid on HeLa cells was more than 10-fold higher than its effect on MCF 7 cells. It is possible that ascorbic acid mediated direct cytotoxicity effects on cancer cells through hydrogen peroxide (Chen et al. 2007). Besides, according to a study by Sant et al. (2018), ascorbic acid promotes apoptosis in breast cancer cells (MDA-MB-231) by increasing TRAIL expression, but the cell type used in their study was non-estrogenic BRCA. Nevertheless, further study is required to elucidate the mechanism of cell death in MCF-7 in the presence of ascorbic acid.

Nonetheless, a high concentration of ascorbic acid does not interfere with chemotherapy or irradiation therapy and may even enhance the efficacy in some situations (Mikirova et al. 2013). It was also reported that ascorbic acid replenishment would protect normal tissues from tumour invasiveness and metastasis due to its role in collagen production and protection.

The result of the MTT assay showed that the combination treatment of ascorbic acid and tamoxifen reduced the proliferation of MCF-7 cells at average concentration compared to tamoxifen alone, which is consistent with the finding of Lee et al. (2019). High doses of vitamin C (1.25 mM to 20.00 mM) were utilised in the study of Lee et al. (2019), whereas this study utilised a fixed concentration of 1.14 mM (202 µg/ml). A fixed concentration of ascorbic acid was used in this study to attain the lowest concentration of tamoxifen required for the optimum effect of ascorbic acid in terms of IC_{50} value. The limitation of this ratio combination design is that the optimal dose of ascorbic acid for combination treatment cannot be determined in this study. However, the lowest dose of tamoxifen determined in this study is useful in reducing the toxicities of tamoxifen in actual treatment.

The combination of ascorbic acid and tamoxifen in this study showed antagonize effect. A previous study suggested that the antagonize effect might be due to some anticancer drugs which mechanism involve reactive oxygen species (ROS) to cause the anticancer effect. Conversely, a highly selective anticancer drug does not require ROS generation (Heaney et al. 2008) to induce anticancer effect. Tamoxifen for example, exhibited antagonize effect via increase oxidative stress through mitochondria-dependent and nitric oxide (NO)-dependent pathways associated with increased intramitochondrial Ca2+ concentration (Bernstein et al. 1999). Besides, the antagonistic effect could be due to the fact that tamoxifen exerts its antineoplastic effect by inducing cell death in HeLa cell via the Fas ligand-mediated mitochondrial membrane depolarization. However, a lower concentration of ascorbic acid inhibits such depolarization (Heaney et al. 2008).

CONCLUSION

In conclusion, tamoxifen was proven to be cytotoxic to both types of cancer cells with an IC_{50} value of 14.53 µg/ml and 11.09 µg/ml, respectively. Conversely, ascorbic acid could only reduce cell proliferation of HeLa cells at a higher concentration (IC_{50} = 202.3 µg/ml). Combination treatment of ascorbic acid and tamoxifen exerted substantial growth reduction (15 %) in MCF-7 cells. In MCF-7 cells, the action of ascorbic acid and tamoxifen was synergistic as all the CI values were below 1. In HeLa cells, the combination of ascorbic acid and tamoxifen treatment exhibited antagonistic action as the CI were more than 1. The inhibition of HeLa cells in combination treatment ranged from 30 % to 70 %. Further studies are required to elucidate the underlying mechanism.

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DISCLOSURE / CONFLICT OF INTEREST\

The authors declare that they have no conflict of interest.

REFERENCES

Aastha Sindhwani, B. C., Sasikala Muthusammy, B. C., & Alka Bhatia, A. C–F. 2019. Vitamin C may exert variable effects on viability and proliferation of HeLa cells exhibiting high and low chromosomal instability. *Advance in Clinical and Experimental Medicine*. 28(1).19–24.

Acharya, S., Hensley, M. L., Montag, A. C., & Fleming, G. F. 2005. Rare uterine cancers. *Lancet Oncology*. ; 6: 961–971. doi: 10.1016/S1470-2045(05)70463-0.

An, Q., Sun, C., Li. D., Xu, K., Guo, J., & Wang, C. 2013. Peroxidase-Like Activity of Fe_3O_4 @ Carbon Nanoparticles Enhances Ascorbic Acid-Induced Oxidative Stress and Selective Damage to PC-3

Prostate Cancer Cells. *ACS Applied Materials & Interfaces*. 5(24). 13248–13257. doi:10.1021/am4042367

Basu, T., Panja, S., Ghate, N. B., Chaudhuri, D. & Mandal, N. 2017. Antioxidant and antiproliferative effects of different solvent fractions from *Terminalia belerica* Roxb. fruit on various cancer cells. *Cytotechnology*. 69(2). 201–216. doi:10.1007/s10616-016-0051-6

Bayat Mokhtari, R., Homayouni, T. S., Baluch, N., Morgatskaya, E., Kumar, S., Das, B. & Yeger, H. 2017. Combination therapy in combating cancer. *Oncotarget*. 8(23). 38022–38043. doi:10.18632/oncotarget.16723

Bernstein, L., Deapen, D., Cerhan, J. R., Schwartz, S. M., Liff, J., McGann-Maloney, E., Perlman, J. A & Ford, L. 1999. Tamoxifen therapy for breast cancer and endometrial cancer risk. *Journal of the National Cancer Institute*. 91. 1654–1662. doi: 10.1093/jnci/91.19.1654.

Bogush, T., Dudko, E., Bogush, E., Polotsky, B., Tjulandin, S., & Davydov, M. 2012. Tamoxifen non-estrogen receptor mediated molecular targets. *Oncology Reviews*. 6(2): e15. doi:10.4081/oncol.2012.e15

Catani, M. V., Costanzo, A., Savini, I., Levrero, M., de Laurenzi, V., Wang, J. Y., Melino, G., & Avigliano, L. (2002). Ascorbate up-regulates MLH1 (Mut L homologue-1) and p73: implications for the cellular response to DNA damage. *The Biochemical journal*, 364(Pt 2), 441–447. https://doi.org/10.1042/BJ20011713

Chen, Q., Espey, M. G., Sun, A. Y., Lee, J. H., Krishna, M.C, Shacter, E., Choyke, P. L., Pooput, C., Kirk, K. L., Buettner, G. R. & Levine M. 2007. Ascorbate in pharmacologic concentrations selectively generates ascorbate radical and hydrogen peroxide in extracellular fluid in vivo. *Proceeding National Academic Science USA*. 104:8749–8754.

Chou, T, C. & Martin, N. 2005. CompuSyn for Drug Combinations and for General Dose-Effect Analysis, User's Guide, ComboSyn, Inc.

Chung, S. H., Franceschi, S., & Lambert, P. F. 2010. Estrogen and ERalpha: culprits in cervical cancer?. *Trends in endocrinology and metabolism: TEM*, 21(8), 504–511. https://doi.org/10.1016/j.tem.2010.03.005

Davies, C., Godwin, J., Gray, R., Clarke, M., Cutter, D., Darby, S., McGale, P., Pan, H. C., Taylor, C., Wang, Y. C., Dowsett, M., Ingle, J. & Peto, R. 2011. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). *Lancet*. 378. 771–784.

Espey, M. G., Chen, P., Chalmers, B., Drisko, J., Sun, A. Y., Levine, M. & Chen, Q. 2011. Pharmacologic ascorbate synergizes with gemcitabine in preclinical models of pancreatic cancer. *Free Radical Biology & Medical*. 50. 1610–1619. doi: 10.1016/j.freeradbiomed.

Fernando, J. & Jones, R. 2015. The principles of cancer treatment by chemotherapy. *Surgery.* 33(3): 131–135.

González, M. J., Rosario-Pérez, G., Guzmán, A. M., Miranda-Massari, J. R., Duconge, J., Lavergne, J., et al. 2010. Mitochondria, energy and cancer: the relationship with ascorbic acid. *J Orthomol Med.* 25(1):29-38.

Greenlee, H., Hershman, D. L. & Jacobson, J. S. 2009. Use of antioxidant supplements during breast cancer treatment: a comprehensive review. *Breast Cancer Research and Treatment*. 115(3). 437–452.

Gröber, U. 2009.Antioxidants and other micronutrients in complementary oncology. Breast Care (Basel). 4(1),13-20.

Guerriero, E., Sorice, A., Capone, F., Napolitano, V., Colonna, G., Storti, G., et al. 2014. Vitamin C effect on mitoxantrone-induced cytotoxicity in human breast cancer cell lines. *PloS One*. 9(12):e115287.

Harris, H. R., Orsini, N. & Wolk, A. 2014. Vitamin C and survival among women with breast cancer: A Meta-analysis. *European Journal of Cancer*. 50(7). 1223–1231.

Heaney, M. L. Gardner, J. R., Karasavvas, N, Golde, D. W., Scheinberg, D. A., Smith, E. A. & O'Connor, O. A. 2008. Vitamin C antagonizes the cytotoxic effects of antineoplastic drugs. *Cancer Research*. 68(19). 8031–8038.

Hwang, J. Y., Lin, B.Y., Tang, F, M & Yu, W. C. 1992. Tamoxifen Stimulates Human Papillomavirus Type 16 Gene Expression and Cell Proliferation in a Cervical Cancer Cell Line. *Cancer Research*. 52(24). 6848-52.

Kaufmann, S. H, & Gores, G, J. 2000. Apoptosis in cancer: cause and cure. *Bioessays*. 22(11). 1007–1017.

Kelly, P., Keely, N., Bright, S., Yassin, B., Ana, G., Fayne, D., Zisterer, D. & Meegan, M. 2017. Novel selective estrogen receptor ligand conjugates incorporating endoxifen-combretastatin and cyclofenil-combretastatin hybrid scaffolds: Synthesis and biochemical evaluation. *Molecules*. 22(9). 1440.

Kumar, S., Chaudhary, A. K., Kumar, R., O'Malley, J., Dubrovska, A., Wang, X. & Chandra, D. 2016. Combination therapy induces unfolded protein response and cytoskeletal rearrangement leading to mitochondrial apoptosis in prostate cancer. *Molecular Oncology.* 20(7). 949–965.

Lee, J. H. & Nan, A. 2012. Combination drug delivery approaches in metastatic breast cancer. *Journal of Drug Delivery*. 915375. doi:10.1155/2012/915375

Lee, S. J., Jeong, J. H., Lee, I. H., Lee, J., Jung, J. H., Park, H. Y., Lee, D. H. & Chae, Y.S. 2019. Effect of High-dose Vitamin C Combined with Anti-cancer Treatment on Breast Cancer Cells. *Anticancer Research*. 39(2):751-758. doi: 10.21873/anticanres.13172. PMID: 30711954.

Mata, A. M., Carvalho, R. M., Alencar, M. V., Cavalcante, A. A. & Silva, B. B. 2016. Ascorbic acid in the prevention and treatment of cancer. *Revista da Associação Médica Brasileira*. 62(7):680-6.

Mikirova, N., Casciari, J., Riordan, N. & Hunninghake, R. 2013. Clinical experience with intravenous administration of ascorbic acid: achievable levels in blood for different states of inflammation and disease in cancer patients*. Journal of Translational Medicine*. 11(1). 191.

Muralikrishnan, G., Amanullah, S., Basha, M. I., Boopalan, S., Vijayakumar, S., & Shakeel F. 2010. Clinical Study Effect of Vitamin C on Lipidperoxidation and Antioxidant Status in Tamoxifen-Treated Breast Cancer Patients. *Chemotherapy*. 56. 298–302.

Nasu, K., Takai, N., Nishida, M., & Narahara, H. 2008. Tumorigenic effects of tamoxifen on the female genital tract. *Clinical medicine. Pathology*, *1*, 17–34. https://doi.org/10.4137/cpath.s487

National Cancer Institue. USA.2020. Drugs approved for the treatment of cervical cancer. https://www.cancer.gov/about-cancer/treatment/drugs/cervical. (23/12/2020)

Negreira, N, Regueiro, J., López de Alda, M. & Barceló, D. 2015. Transformation of tamoxifen and its major metabolites during water chlorination: Identification and in silico toxicity assessment of their disinfection by products. *Water Research*. 85. 199–207.

Notas, G., Pelekanou, V., Kampa, M., Alexakis, K., Sfakianakis, S., Laliotis, A. & Castanas, E. 2015. Tamoxifen induces a pluripotency signature in breast cancer cells and human tumors. *Molecular Oncology*. 9(9). 1744–1759.

Qiao, H., Wang, T-Y., Yan, W., Qin, A., Fan, Q-M., Han, X-G. & Tang T-T. 2015. Synergistic suppression of human breast cancer cells by combination of plumbagin and zoledronic acid in vitro. *Acta Pharmacologica Sinica*. 36. 1085–1098.

Rawal, M., Schroeder, S. R., Wagner, B. A., Cushing, C. M., Welsh, J. L., Button, A. M., Du, J., Sibenaller, Z. A., Buettner, G. R. & Cullen, J. J. 2013. Manganoporphyrins increase ascorbate-induced cytotoxicity by enhancing H2O2 generation. *Cancer Research.*. 73(16). 5232-41.

Reid, J. M., Goetz, M. P., Buhrow, S. A., Walden, C., Safgren, S. L., Kuffel, M. J., Reinicke, K. E., Suman, V., Haluska, P., Hou, X., Ames, M. M. 2014. Pharmacokinetics of endoxifen and tamoxifen in female mice: implications for comparative in vivo activity studies. *Cancer Chemotherapy and Pharmacology*. 74(6). 1271–1278.

Rivera-Guevara, C., Pérez-Alvarez, V., García-Becerra, R., Ordaz-Rosado, D., Morales-Ríos, M. S., Hernández-Gallegos, E., Cooney, A. J., Bravo-Gómez, M. E., Larrea, F. & Camacho, J. 2010. Genomic action of permanently charged tamoxifen derivatives via estrogen receptor-α. *Bioorganic & Medicinal Chemistry*. 18(15). 5593–5601.

Sant, D. W., Mustafi, S., Gustafson, C. B., Chen, J., Slingerland, J. M. & Wang, G. 2018. Vitamin C promotes apoptosis in breast cancer cells by increasing TRAIL expression. Scientific Reports. 8(1). 5306.

Stephenson, C. M., Levin, R. D., Spector, T. & Lis, C. G. 2013. Phase I clinical trial to evaluate the safety, tolerability, and pharmacokinetics of high-dose intravenous ascorbic acid in patients with advanced cancer. *Cancer Chemotherapy and Pharmacology*.; 72(1):139–146.

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A. & Bray, F. (2021) Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer Journal for Clinician*. 0: 1-41.

Unlu, A., Kirca, O., Ozdogan, M., Nayır, E. 2016. High-dose vitamin C and cancer. *Journal of Oncological Science*. 1. 10–12.

van Leeuwen, F. E., Van den Belt-Dusebout, A. W., Benraadt J, Diepenhorst, F. W., Van Tinteren, H., Coebergh, J. W., Kiemeney, L. A., Gimbrère, C. H., Otter, R., Schouten, L. J. & Damhuis, R. A. 1994. Risk of endometrial cancer after tamoxifen treatment of breast cancer. *The Lancet*. 343(8895). 448-452.

Wan Omar, W. A., Azhar N. A., Harif Fadzilah, N. & Nik Mohamed Kamal, N. N. S. 2016. Bee pollen extract of Malaysian stingless bee enhances the effect of cisplatin on breast cancer cell lines. *Asian Pacific Journal of Tropical Biomedicine*. 6(3). 265–269.

Wang, L., Luo, X., Li, C., Huang, Y., Xu, P., Lloyd-Davies, L. H. & Baker P. 2017. Triethylenetetramine Synergizes with Pharmacologic Ascorbic Acid in Hydrogen Peroxide Mediated Selective Toxicity to Breast Cancer Cell. *Oxidative Medicine and Cellular Longevity*. 1–13.

Yaacob, N. S., Nengsih, A. & Norazmi, M. N. 2013. Tualang Honey Promotes Apoptotic Cell Death Induced by Tamoxifen in Breast Cancer Cell Lines. *Evidence-Based Complementary and Alternative Medicine.* 1–9. doi:10.1155/2013/989841.

Yeom, C-H., Lee, G., Park, J-H., Yu, J., Park, S., Yi, S-Y. & Lee, S. 2009. High dose concentration administration of ascorbic acid inhibits tumor growth in BALB/C mice implanted with sarcoma 180 cancer cells via the restriction of angiogenesis. *Journal of Translational Medicine*. 7(1). 70.

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