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Combined Caffeine and Cisplatin Treatment Induces Synergistic Cytotoxicity in Hela Cell Line

Saeb H. Aliwaini[1,*], Sanabel A. Dawas[1], Husam Eddeen M. Abu Tayem[1], Salsabeel H. Aljoujou[1],

[1]Department of Biology and Biotechnology, Faculty of Science, Islamic University of Gaza, Gaza Strip, Palestine, [1]Department of Medical Laboratory Sciences, Faculty of Applied Medical Sciences, Islamic University of Gaza, Gaza Strip, Palestine

*** Corresponding author**

E-mail address: siwini@iugaza.edu.ps

Abstract

Cisplatin is a common alkylating anticancer agent that has been used to treat several cancers. However, the efficiency of cisplatin treatment is limited due to the severe side effects and the resistance to the drug, which eventually results in treatment failure. Caffeine is a natural ingredient contained in many food sources. Caffeine has been shown to induce cell cycle arrest and apoptosis in different cancer cell types. The effect of caffeine on cisplatin treatment on cervical cancer cells is not well known. Here we examined the combined effect of caffeine and cisplatin in human Hela cells. The cancer cells were exposed to different concentrations of caffeine and cisplatin and IC_{50}'s were determined by MTT assay. Cell number and viability were measured by cell counting and trypan blue assays. Data obtained show that, caffeine treatment enhances the anti-proliferation effect of cisplatin and lowered the IC_{50} of cisplatin from 8.93 µM to be 2.75 µM. These results suggest that caffeine-assisted chemotherapy is useful for cervical cancer treatment.

Keywords:
Cervical cancer,
Cisplatin,
Caffeine.

1. Introduction:

Cervical cancer is one of the common cancers in women worldwide. According to global statistics, an estimated 12,990 cases of invasive cervical cancer were diagnosed, and 4,120 patients died of this disease in 2016 (American Cancer Society, 2016). Cervical cancer can be treated by different therapeutic strategies such as surgery, chemotherapy, radiation therapy, hormonal therapy, and targeted therapy. The choice of therapy depends upon the location, grade of tumor and stage of disease, as well as the general state of the patient (Belachew, Erku, Mekuria, Melaku, & Gebresillassie, 2016).

Chemotherapy, anti-neoplastic therapy and cytotoxic therapy are three medical terms used to describe chemical agents used in cancer therapy but the most commonly used is chemotherapy. Unlike surgery and radiation, chemotherapy is used as a systemic approach to treat cancer and is especially important for patients with advanced stages of cancer. Currently, more than 100 chemotherapeutic agents are used either as single treatment or in combination with other treatments. Platinum

analogues such as cisplatin and carboplatin, as well as other alkylating agents are still frequently used chemotherapies for several cancers (Leisching, Loos, Botha, & Engelbrecht, 2015). To date, cisplatin ovarian, cervical, head and neck, and non-small-cell lung cancer (Jamieson & Lippard, 1999). It is particularly successful for the treatment of testicular cancer with approximately 100% cure rate if tumors are detected early (Bruijnincx & Sadler, 2008). It is widely accepted that cisplatin cytotoxicity is initiated by forming DNA adducts and subsequently blocking replication and/or preventing transcription (D. Wang & Lippard, 2005). The most common types of cell death induced by cisplatin are apoptosis and necrosis (D. Wang & Lippard, 2005). The effectiveness of cisplatin (CDDP)against most tumors, however, declines severely due to tumor-acquired resistance and the high dose of cisplatin used has been associated with severe side-effects such as neuro-, hepato- and nephron-toxicity (Jung & Lippard, 2007). Huge efforts to overcome the limitations of cisplatin treatments have been made and one of the suggested therapeutic strategies is drug combination strategy (G. Wang, Bhoopalan, Wang, Wang, & Xu, 2015). In this regard, cisplatin is commonly used in combination with some other drugs for cancer treatment (Deng, Zhang, & Chen, 2013; Du et al., 2013). Generally, drug combinations have better therapeutic outcomes than single anticancer drug taking into consideration make a good balance between drug activity and toxicity (D. Lu, Lu, & Cao, 2013). Therefore, researchers are looking for natural and safe combinations to get better anticancer effects with very little side effects. In this regard, several studies showed that caffeine (natural purine alkaloid found in coffee, tea, and cacao) can induce biological effects such as apoptosis and autophagy in different cancer cells (Bøhn et al., 2014; G.-Y. Lu et al., 2014). Importantly, caffeine has also been shown to inhibit ATM and ATR -two important protein kinases involved in DNA damage– and to induce cell cycle arrest/apoptosis signaling process (F. C. Wang, Wu, & Geim, 2015). The current study therefore studied the potency of caffeine as anticancer natural product as well as its ability to enhance anticancer effect of cisplatin. Results obtained from this study showed that treatment with moderate caffeine concentrations inhibits Hela cells proliferation and small concentrations of caffeine strongly enhance cisplatin anticancer effect.

remains one of the most important chemotherapeutic agents and has been used extensively to treat several cancers including breast,

2. Material and methods:

2.1 Cell line and cell culture:

Hela cell line used in the studies obtained from Birzeit University in Palestine (Dr. Johnny Stiban). Hela cell line is human cervix epithelial carcinoma cells derived from a cervical carcinoma from a 31 years old female. These cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin antibiotic and cultured at 37°C with 5% CO_2.

2.2 Caffeine and cisplatin treatment:

Both caffeine and cisplatin were purchased from Middle East Pharmaceutical Company in Gaza. Caffeine was prepared as 1000 mM stock in sterile distilled water, dissolved at 80°C to become clear and stored at −20°C. Cisplatin was freshly prepared as a 1 mM solution in DMEM media and stored at room temperature. Cells were seeded and cultured at 37°C for 24 hours to reach approximately 70% confluence, then treated with caffeine and cisplatin for 48 hours.

2.3 Cytotoxicity Assay:

Cytotoxicity of treatment on Hela cells was measured by microculture tetrazolium (MTT) assay (Mosmann, 1983). For this assay, 96-well microplates were seeded with 100 µl medium containing 5000 cells to reach approximately 60% confluence after 24 hours of incubation. The cells were then treated with different concentrations from caffeine stock 1000 mM, and cisplatin stock 1mM. After 48 hours incubation, cell viability was determined by adding 10µl MTT (3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) from sigma and incubated for 4 hours. Then we added 100 µl of solubilizing solution (sigma) for 16 hours incubation and reading the plate at 620 nm absorbance by Elisa reader (Multiskan™ FC Microplate, Thermo, USA). Tetrazolium salts are cleaved to formazan dye by cellular enzymes (only in the viable cells). The level of absorbance directly correlates to the metabolically active cells.

2.4 Cell viability assay:

For cell viability quantification, cells were washed with phosphate-buffered saline, disaggregated by trypsin and collected as single cell suspensions then it was stained with 1:2 Trypan blue stain. Total and non-viable cells were manually determined using counting chamber.

3. Results:

3.1 Cisplatin induces moderate cytotoxic effect against Hela cells:

Previous studies showed that cisplatin induces anti-proliferating effect against Hela cells with an IC_{50} less than 10 µM (Abdul et al., 2008; Minagawa, Kigawa, Itamochi, & Kanamori, 1999). To confirm these results in our conditions the cytotoxic effect of cisplatin on the Hela cells was examined using the MTT assay. After 48 hours of treatment with cisplatin, a dose dependent inhibition of cell proliferation was observed (Figure 1). Indeed, the IC_{50} value obtained was 8.93 µM as calculated from three different experiments. Importantly 15 µM of cisplatin killed about 70 % of cervical cancer cells and 100% cell death was not achieved using these concentrations. These results show that cisplatin exerts moderate cytotoxic effects against cervical cancer cells in vitro.

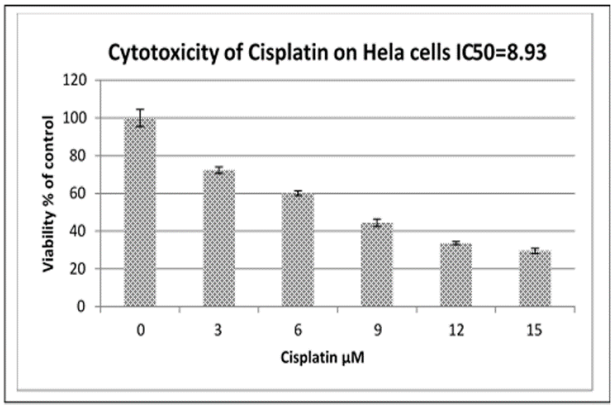


Figure 1 *Cisplatin induces cytotoxicity in cervical cancer cells*

Cell survival rate (as measured by MTT assay) of indicated cell line treated with increasing concentrations of cisplatin (0-15 µM) or vehicle for 48 hours. Results show the mean percentage ± SEM of untreated cells and represent the pooled results of at least three experiments performed in quadruplicate. The concentration of cisplatin required for killing 50% of the cells (IC_{50}) is shown.

3.2 Caffeine effect on Hela cells proliferation:

Earlier reports indicated that caffeine might play anti-proliferating effects against different cancer cells (Alao & Sunnerhagen, 2009; Blasina, Price, Turenne, & McGowan, 1999; G.-Y. Lu et al., 2014; Ohsaki et al., 1990; Saiki et al., 2011). To study the possible effect of caffeine on Hela cells, cells were plated and treated with different concentrations of caffeine (0-10 µM) and proliferation rate examined using the MTT assay. After 48 hours of caffeine treatment, a dose dependent inhibition of cell proliferation observed (Figure. 2). Indeed, the IC_{50} value obtained was 7.18 µM as calculated from three different experiments. Importantly, while 10 µM of caffeine killed about 60 % of cervical cancer cells 2 µM did not show a potent cytotoxic effect. These results show that only high doses of caffeine exert cytotoxic effects against cervical cancer cells in vitro. Therefore, we next moved to test the cytotoxic effect of the combined treatment of cisplatin with low concentrations of caffeine.

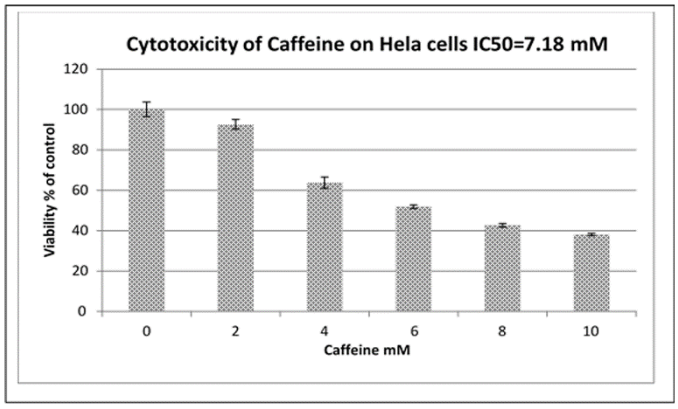


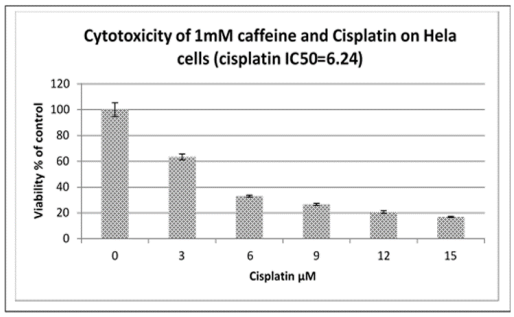
Figure 2 *Caffeine inhibits cervical cancer growth in vitro*

Cell survival rate (as measured by MTT assay) of Hela cells treated with different concentrations of caffeine (0-10 mM) or vehicle for 48 hours. Results display the mean percentage ± SEM of untreated cells and represent the pooled results of at least three experiments performed in quadruplicate. The concentration of caffeine required for killing 50% of the cells (IC_{50}) is shown.

3.3 Cisplatin and caffeine synergistically inhibit Hela cell survival:

To determine whether combined treatment of cisplatin and caffeine may have a greater anti-cancer effect on Hela cells than single treatment, cells were treated with a low dose of caffeine 1 mM and different concentrations of cisplatin (0-15 µM). This dose of caffeine was chosen based on the MTT results which showed that 2 mM of caffeine exerts a small level of toxicity. Figure 3a shows that 1mM of caffeine enhances cisplatin cytotoxicity and resulted with new IC_{50} of 6.24 µM of cisplatin instead of 8.93 µM of cisplatin when cells was treated with cisplatin only. These results show that 1mM of caffeine slightly enhances cisplatin cytotoxicity. In view of these data, we wondered whether 2 mM of caffeine further enhances cisplatin cytotoxicity. To this end, cells were treated with 2 mM of caffeine and increasing concentrations (0-5 µM) of cisplatin. Figure 3b demonstrates that the combined treatment resulted in enhanced cytotoxic activity with a lower **IC_{50} of 2.75** µM cisplatin. These data show that caffeine /cisplatin combined treatment results in a synergistic cytotoxic effect in cervical cancer cells.

a



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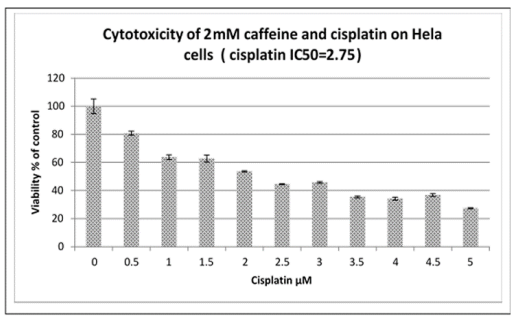

Figure 3 *Caffeine enhances cisplatin cytotoxicity on cervical cancer growth in vitro*

Cell survival rate (as measured by MTT assay) of Hela cells treated with; (a) different concentrations of cisplatin (0-10 µM) or vehicle with caffeine 1 mM for 48 hours and in (b) different concentrations of cisplatin (0-5 µM) or vehicle with caffeine 2 mM for 48 hours. Results display the mean percentage ± SEM of untreated cells and represent the pooled results of at least three experiments performed in quadruplicate. New cisplatin IC_{50}'s in the presence of the indicated concentrations of caffeine is shown.

3.4 Combined treatment of cisplatin and caffeine exerts its effect by inducing a cell death mechanism in Hela cells:

We next aimed to investigate the mechanism by which combined cisplatin and caffeine treatment synergistically inhibits Hela cells survival. To this end, Hela cells were treated with vehicle, cisplatin (3.0 µM), caffeine (2.0 mM) or cisplatin-caffeine (3.0 and 2.0 mM, respectively) and the effect on the cell viability and proliferation rate were tested by trypan blue and cell counting. Figure 4a shows that combined cisplatin – caffeine treatment induced a significantly inhibition of cell viability about 60% inhibition while the same treatment in Figure 4.b resulted in more than 70% decrease in cell number in comparison to the untreated Hela cells. These results suggest that the combined treatment of cisplatin and caffeine induces a cell death mechanism, which may explain the decrease in cell number also. To confirm these results morphological studies were also performed and results in Figure 5 shows that Hela cells treated with this combination is extremely stressed with high level of floating cells, which might represent the dead cells. Together, all these results show that caffeine enhances the cytotoxic effect of cisplatin against Hela cells in vitro mainly by inducing a type of cell death mechanism.

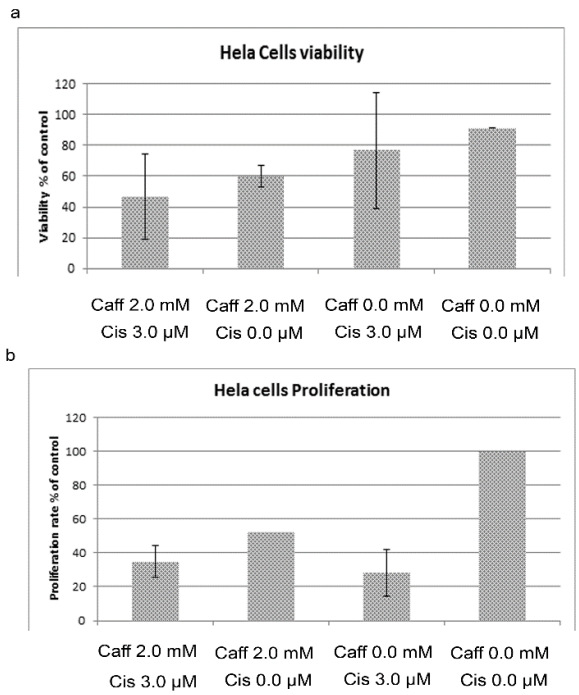

Figure 4 *Caffeine enhances cisplatin cytotoxicity by inducing cell death in cervical cancer*

(a) Cell viability rate (as measured by trypan blue assay) of Hela cells treated with cisplatin 3 µM, caffeine 2 mM, caffeine 2 mM-cisplatin 3 µM or vehicle for 48 hours. (b) Cell proliferation rates (as measured by cell counting) of Hela cells treated with cisplatin 3 µM, caffeine 2 mM, caffeine 2 mM-cisplatin 3 µM or vehicle for 48 hours. Results display the mean percentage ± SEM of untreated cells and represent the pooled results of at least three experiments performed in quadruplicate.

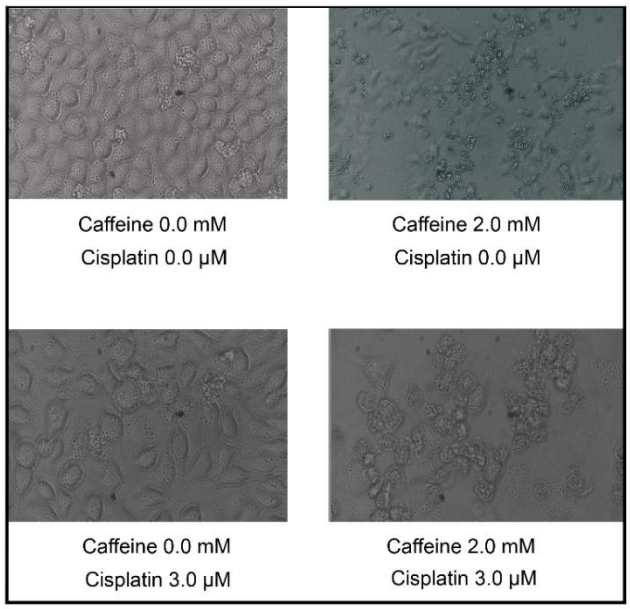

Figure 5 *Caffeine- cisplatin combined treatment induces apoptotic morphological changes in Hela cells*

Representative photomicrographs (400x) of Hela cells treated with cisplatin 3 µM, caffeine 2 mM, caffeine 2 mM-cisplatin 3 µM or vehicle for 48 hours.

4. Discussion:

Caffeine is one of the most common natural neuro-active compounds which has been shown to induce cytotoxicity in different cancer cell lines (Blasina et al., 1999; Kawano et al., 2012; G.-Y. Lu et al., 2014; Saiki et al., 2011). Caffeine-assisted chemotherapy has been studied in some cancers such as osteosarcomas, gastric, lung and breast cancer (Hayashi et al., 2005; Kawano et al., 2012; Takahashi et al., 1998; G. Wang et al., 2015). Earlier study revealed that caffeine treatment in combination with cisplatin prolonged the survival time of gastric cancer mice than cisplatin alone (Takahashi et al., 1998). However, this study did not describe the mechanism by which caffeine might play its anticancer role. More studies highlighted the effect of caffeine in inducing apoptosis mainly by inhibition of ATR / ATM (DNA damage response kinases). These findings were supported by other studies indicated that ATR/ATM inhibition accelerates apoptosis and cell death (Heffernan et al., 2009). Another study reported that caffeine plays a role in autophagy activation and AKT inhibition (Saiki et al., 2011). Importantly, this study showed that autophagy, at least in this case, is a pro apoptotic mechanism (Aliwaini et al., 2015; Aliwaini,

Bleloch, Kimani, & Prince, 2016; Aliwaini, Swarts, Blanckenberg, Mapolie, & Prince, 2013). In agreement with all these previous studies, we show here that caffeine as a single treatment has a cytotoxic effect against Hela cells. While the current study did not show how caffeine exerts this effect precisely, we show that caffeine treatment decreases the percent of viable cells and increases the dead cells. It's important to note that most of the previous studies used caffeine in a relatively high concentration more than 10 mM to induce its effect which indicate a minor cytotoxic effect of caffeine at low doses (Hayashi et al., 2005; Heffernan et al., 2009). Therefore, several studies suggested using caffeine with different concentrations to enhance the cytotoxic effect of different chemotherapeutic agents such as cisplatin. Cisplatin was shown to induce its effect against Hela cells in a moderate concentrations more than 5 µM (Andreu-Fernández et al., 2013). In accordance with this, our results also show that cisplatin has an IC_{50} of 8.93 µM on Hela cells. Notably, we show here that caffeine treatment with a low dose 2 mM enhances cisplatin cytotoxicity, which seems to be by inducing cell death. All together this findings support previous studies on the use of caffeine in the treatment of human tumors.

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الكافيين يعزز سمية علاج السيسبلاتين على خلايا سرطان عنق الرحم

يعتبر عقار السيسبلاتين أحد أشهر مضادات للسرطان الا أن فعالية السيسبلاتين محدودة بسبب الاثار الجانبية الشديدة والمقاومة التي يبديها السرطان لهذا العقار مما يؤدي في نهاية المطاف إلى فشل العلاج. الكافيين هو مركب طبيعي موجود في العديد من المصادر الغذائية. وقد اشارت الدراسات السابقة على قدرة الكافيين على وقف دورة الخلية السرطانية واحداث الموت المبرمج فيها. ومع ذلك فإن تأثير الكافيين على فعالية علاج سيسبلاتين على خلايا سرطان عنق الرحم ليست معروفة جيدا. هذه الدراسة عنيت بفحص التأثير المشترك للكافيين والسيسبلاتين في خلايا سرطان عنق الرحم. وفي الدراسة تم تعريض الخلايا السرطانية لتركيزات مختلفة من الكافيين والسيسبلاتين للحصول على IC50 وذلك بطريقة MTT وبطريقة التريبان الازرق. وبينت النتائج أن الكافيين يعزز التأثير المضاد للسرطان لعقارالسيسبلاتين وخفضت IC50 للسيسبلاتين من 8.93 ميكروومولر لتكون 2.75 ميكروومولر. وتشير هذه النتائج إلى أن العلاج الكيميائي بمساعدة الكافيين قد يفيد للعلاج من سرطان عنق الرحم.

كلمات مفتاحية:
الكافيين
السيسبلاتين
سرطان عنق الرحم
الموت المبرمج