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Evaluation of Antineoplastic Drugs in Cervix Cancer Spheroids

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ABSTRACT

Cervix cancer is the second most common cancer among Mexican female population. Current chemotherapeutic regimens used in clinical settings are associated with substantial toxicity and low efficacy. Therefore, it is necessary to explore new therapies and use drugs in cytotoxic and cytostatic combinations. An ideal in vitro model for the study of drugs is tumor spheroid culture. The model retains the important features and functionalities of the tumor in vivo, allowing for more efficient identification of drugs that influence cancer cells. This study aimed to evaluate four antineoplastic drugs (cisplatin, 5-fluorouracil, paclitaxel, and doxorubicin) used in the treatment of solid tumors in spheroids formed from cells of patients diagnosed with cervix cancer. Cytotoxicity and apoptosis during the interaction with antineoplastic agents were evaluated, and dose-response curves were constructed for each drug using checkerboard tests, thus evaluating the synergism between pairs of drugs. We obtained an efficacy of 71% for spheroid formation, and the best treatment was with doxorubicin, a drug that significantly reduced cell viability ($p=0.001$). However, the construction of dose-response curves showed variations in drug sensitivity for each patient. We can conclude that each spheroid responded differently to the treatments administered, suggesting that personalized treatment could improve the response to chemotherapy in cervix cancer patients.

Keywords: 3D cell model, Tumor spheroids, Antineoplastic drugs, Cervix cancer.

Introduction

Among female genital tumors, cervix cancer is the second most common cancer in Mexican females [1]. It has been highlighted in recent years that cervix cancer is on the rise; globally, approximately 500,000 new cases of cervix cancer are diagnosed, and approximately 80% correspond to low-income countries [2-5].

Owing to the high incidence and mortality rates, innovative, affordable, efficient, and effective approaches [6, 7]. Regarding chemotherapeutic treatment, patients respond differently to drug therapy because of genetic, epigenetic, and environmental factors that affect the proteins that metabolize or transport drugs, their therapeutic targets (receptors), or both; the contribution of each factor varies for each drug. Consequently, new approaches have emerged, including the development of new drugs, forms of chemotherapy, and drug delivery systems [8, 9]. The design and development of new drugs does not have rapid advances to meet clinical needs, so there are other alternatives, such as drug repositioning [10, 11]. Conducting new research with drugs already used in the clinic ensures cost and time reduction because the safety and pharmacokinetic profiles are known, which reduces the risk compared to other strategies.

One promising approach is to test the therapeutic response of cancer cells obtained from a patient's tumor to various drugs [12]. Viable cancer cells can be isolated from freshly obtained tumor tissue, maintained viable, cultured under *in vitro* conditions, and subsequently exposed to therapeutic drugs[13]. Solid tumor models useful for drug studies include multicellular tumor spheroids [14, 15]. One of the aspects that the culture model allows for drug penetration studies, as it has the potential to evaluate therapeutic response quickly and at a lower cost than other models, is that the spheroid culture can remain viable for up to 21 days [13, 16]. Allowing long-term assays while preserving important features and functionalities of the original tumor [17].

In this case, with the implementation of spheroid cultures and the evaluation of antineoplastic drugs with different mechanisms of action, we obtained a specific picture of each patient's response. This information is useful for overcoming the limitations of current therapies, and we can also support specific treatments according to individual responses, ensuring a better response to treatment.

Methodology

1. STUDY APPROVAL

The samples were collected from the tumor bank of the Mexican National Institute of Cancer (http://www.incan.salud.gob.mx/. The samples provided informed consent and were authorized by the patient from whom biopsy was performed. The Protocol was approved by the Ethics and Research Committee of the Hospital (number 012/048/IMO / CB/20T). The processing and analysis of the samples were carried out in Coordination for the Innovation and Application of Science and Technology (CIACYT), Innovation Unit in Cellular and Molecular Diagnosis, Autonomous University of San Luis Potosí.

2. TUMOR SPHEROID CULTURE FROM CACU PATIENT SPECIMENS

Samples were obtained from patients with CaCu (CaCu *In situ* according to the FIGO classification), as mentioned above, and the protocol was approved by the hospital's Ethics and Research Committee under number 012/048/IMO / CB/20T. Cell counting was performed using a Neubauer Chamber. The appropriate cell density was established to seed 200 μL of the original sample in a 96-well Corning® spheroid microplates Ultra-Low Attachment surface (ULA) box, and the cells were seeded in DMEM medium with 10% fetal bovine serum (FBS) and 1% antibiotic (penicillin) at 37 °C. 5% CO flow$_2$ for 72 h. Spheroidal culture was determined when regular borders without clearly defined individual cells were visualized. Spheroid formation was observed using an inverted microscope.

3. DAPI STAINING

DAPI (4', 6-diamidino-2-phenylindole) staining was used to visualize cellular organization in the spheroids. DAPI dye is capable of staining the nuclei of living cells and accessing cellular DNA where it is highly avid to the nitrogenous base's adenine and thymine. Spheroids formed by stage *IIB* human cervix cancer cells were used in this study. DAPI dye was added to the 96-well Corning® spheroid microplates ULA (Ultra-Low Attachment surface) plate to incubate for 5 minutes at 37° protected from light. Staining was observed under a 358/461 nm fluorescence microscope.

4. VIABILITY AND APOPTOSIS ASSAYS

Viability and apoptosis were determined using the ApoLive-GloTM Multiplex kit, which measures the number of viable cells as a marker of cytotoxicity and caspase activation as a marker of apoptosis to determine the mechanism of cell death. The first part of the assay measured the activity of a protease marker of cell viability. The second part of the assay used Caspase-Glo, a luminogenic caspase-3/7 substrate, to detect caspase activation, a key biomarker of apoptosis. Spheroids were evaluated by interaction with cisplatin, 5-fluorouracil, paclitaxel, and doxorubicin in a 2.5×10^4 96-well plate, each with at least one spheroid; one was seeded for the control and three for each of the drug concentrations to be tested (10, 50, 100 μM/L). Cytotoxicity was evaluated using the ApoLive-Glo kit, and dose-response curves were constructed for each drug. The assays were performed in triplicate.

5. LDH TEST

Increased LDH activity leads to various types of malignancies, serves as a marker for stem cell cancer, and correlates with poor prognosis. The activity of this enzyme was determined using the Cayman LDH Cytotoxicity assay kit. Lactate dehydrogenase is a soluble cytosolic enzyme that is released into the culture medium after loss of membrane integrity due to compound-induced cytotoxicity. Once spheroids were formed, 50μL of the ALDH enzyme was added to a 96-well plate, after which absorbance readings were taken in a spectrophotometer at 450 nm.

6. ASSAY TO DETERMINE ADDITION SYNERGISM AND ANTAGONISM

Previously, the Inhibitory Fractional Concentration (IFC) of each component was determined based on individual EC50 values. The assay was performed in 96-well plates containing spheroids diluted with two different drugs, and the plates were incubated at optimal temperature and time conditions. These contained concentrations (μM/L) that included values higher and lower than the EC50 previously determined for each drug.

7. STATISTICAL ANALYSIS

GraphPad Prism V8 statistical program was used to analyze the results. We considered p values: ***p=0.001, **p=0.01, and *p=0.05. To determine whether drug exposure time influenced cell viability and apoptosis, a two-way ANOVA statistical test was used. Dose-response curves were constructed to determine the effective concentration (EC_{50} of each drug. For the ALDH release assays, ANOVA with Tukey's post hoc test was used.

Results

1. TUMOR SPHEROID CULTURE FROM CACU PATIENT SPECIMENS

Different densities were tested to determine whether human cervix cells could form spheroids in culture. Human cervix cancer cells were cultured at the cancer stage *in* situ and stage *IIB*. cell density is shown in this study as the number of cells that must be seeded at the end of the spheroid formation process to obtain a sphere with smooth rounded edges without clearly defined individual cells. In the first case (Figure 1-A), 20,000 cells per well were seeded from a sample of *CaCu in situ* and incubated under controlled conditions for 72 h, and the formation of spheroids with rough and irregular circumferences was observed. In the second case (Figure 1-B), 2'000 cells were seeded per well, the plate was incubated under controlled conditions for 72 h, and the formation of spheroids with uniform morphology and rounded and smooth surfaces was observed. In some wells, formation of more than one spheroid was observed. The optimal cell density for our assay was determined to be 2'000 cells per well.

2. DAPI STAINING

Once the cell density was standardized, DAPI staining was performed and spheroids were cultured using the aforementioned methodology. After 96 h of treatment with PTX (Figure 1C), the nuclei were observed to stain uniformly, the formation of a layer of proliferating cells was corroborated, the edges were rounded and smooth, and the intensity in blue was marked on the edges of the sphere, indicating that, with the treatment, the cells in the superficial layer began to be compromised. Spheroids with 96 h of CDDP treatment (Figure 1D) were observed with stained nuclei; the spheroidal shape was present, the edges were diffuse, and the intensity in blue was fainter than in the previous figure, indicating that the treatment does not exert the same cellular damage in this case.

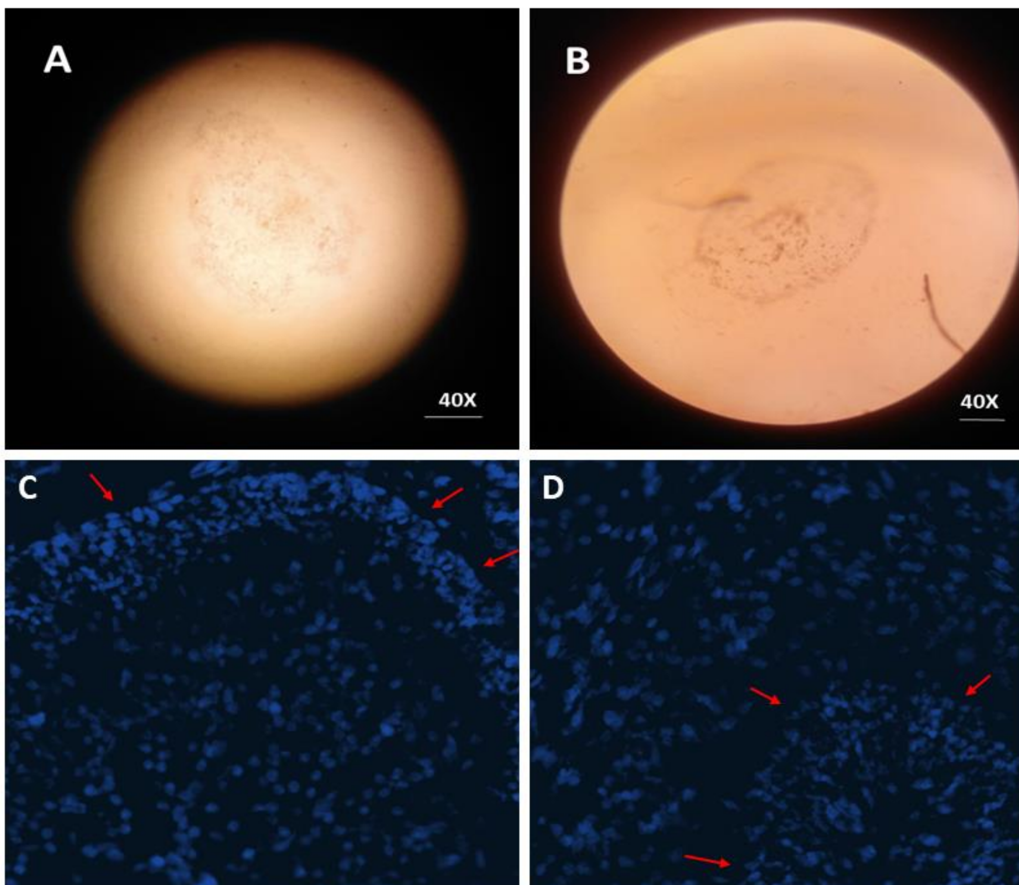

Figure 1. 1A) Spheroid formed from 20'000 cells of human cervix *cancer in situ*, an amorphous figure with irregular edges is observed. 1B) spheroid formed from 2'000 cells of the human cervix in *situ, a* spheroidal figure with regular edges is observed. Inverted field microscope 40X Leica Thermo Fisher. 1C) Spheroid formed from stage *IIB* cervix cancer, 96 hours of culture. Arrows in red show the surface layer of the spheroid, nuclei in blue, and DAPI staining. 1D) Spheroid formed from stage *IIB* cervix cancer, 96 hours of treatment with Cisplatin. Arrows in red show the spheroidal morphology. Nuclei in blue, DAPI staining.

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3. VIABILITY AND APOPTOSIS ASSAYS

A. Evaluation of the viability of spheroids treated with four antineoplastic drugs.

To determine whether drug exposure time influenced spheroid viability, we performed viability assays at four time points. The spheroids were treated with CDDP, 5-FU, PTX, and DXR at concentrations of 100 μM for 24, 72, 96, and 120 h. We also determined whether there were significant differences in the cultures of the drugs used. Statistical analysis was performed using the two-way ANOVA test, and it was observed that the time of exposure to the drugs was not significant; however, DXR showed a significant difference (p=0.001) in inhibiting cell viability compared to the other drugs used. Figure 2 A.

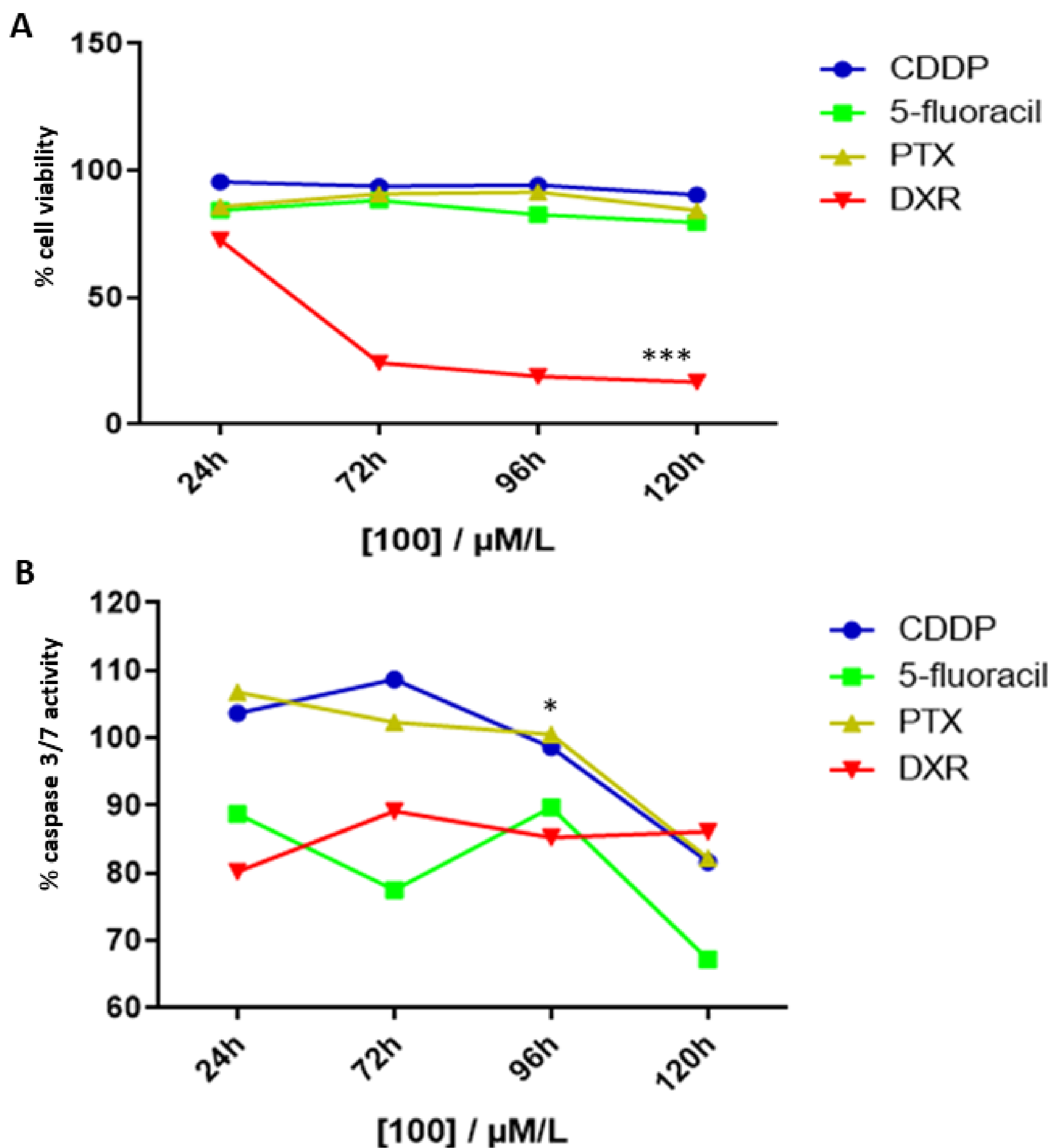

Figure 2. 2A. Viability and Apoptosis on Spheroids of cells cancer. Spheroids were treated with CDDP, 5-FU, PTX, and DXR at a concentration of 100 μM for 24, 72, 96, and 120 hours. Statistical analysis was performed with the two-way ANOVA test, and it was observed that the time of exposure to the drugs was not significant; however, DXR showed a significant difference (p=0.001) in inhibiting cell viability compared to the other drugs used. **2B.** We evaluated the activity of caspases 3 and 7 (Apolive-Glo kit) in spheroids treated with the proposed drugs at a concentration of 100 μM at four different time slices (24, 72, 96, and 120 hours). The statistical analysis also showed a significant decrease (p=0.05) in caspase activity according to the drug used.

B. Evaluation of caspase 3-7 activity in spheroids treated with antineoplastic drugs.

By treating spheroids with the proposed drugs, we aimed to damage the cancer cells, leading to cell death. The activation of the apoptosis cascade involves two pathways: extrinsic and intrinsic. Both pathways converge in the activation of effector caspases 3 and 7. These are responsible for activating deoxyribonuclease, which leads to DNA fragmentation, and therefore, apoptosis.
We evaluated the activity of caspases 3 and 7 (Apolive-Glo kit) in spheroids treated with the proposed drugs at a concentration of 100 μM at four different time points (24, 72, 96, and 120 h). We used a two-way ANOVA and observed that caspase activity started to decrease

significantly (p=0.05) after 96 h of treatment. In contrast, statistical analysis showed a significant decrease (p=0.05) in caspase activity according to the drug used. Figure 2B.

4. ALDH TEST

We performed ALDH release assays using spheroids treated with different concentrations of CDDP, 5-fu, PTX, and DXR. The results show that 5-fu, PTX, and DXR caused no significant changes in the ALDH release. However, spheroids treated with different concentrations of CDDP showed a significant decrease in ALDH levels. Figure 3.

5. ASSAY TO DETERMINE ADDITION SYNERGISM AND ANTAGONISM

A. Effective Concentrations$_{50}$ of antineoplastic drugs on CaCu spheroids.

Dose-response curves were constructed for each drug, and we evaluated the ECs$_{50}$ of the patient spheroids. We compared these values with those previously reported in the literature, which consisted of ECs$_{50}$ determined in cell lines cultured in spheroids with treatment times similar to ours. For CDDP we observed ECs$_{50}$ similar to those reported in the literature for both cases (CaCu *in situ, IIB*).

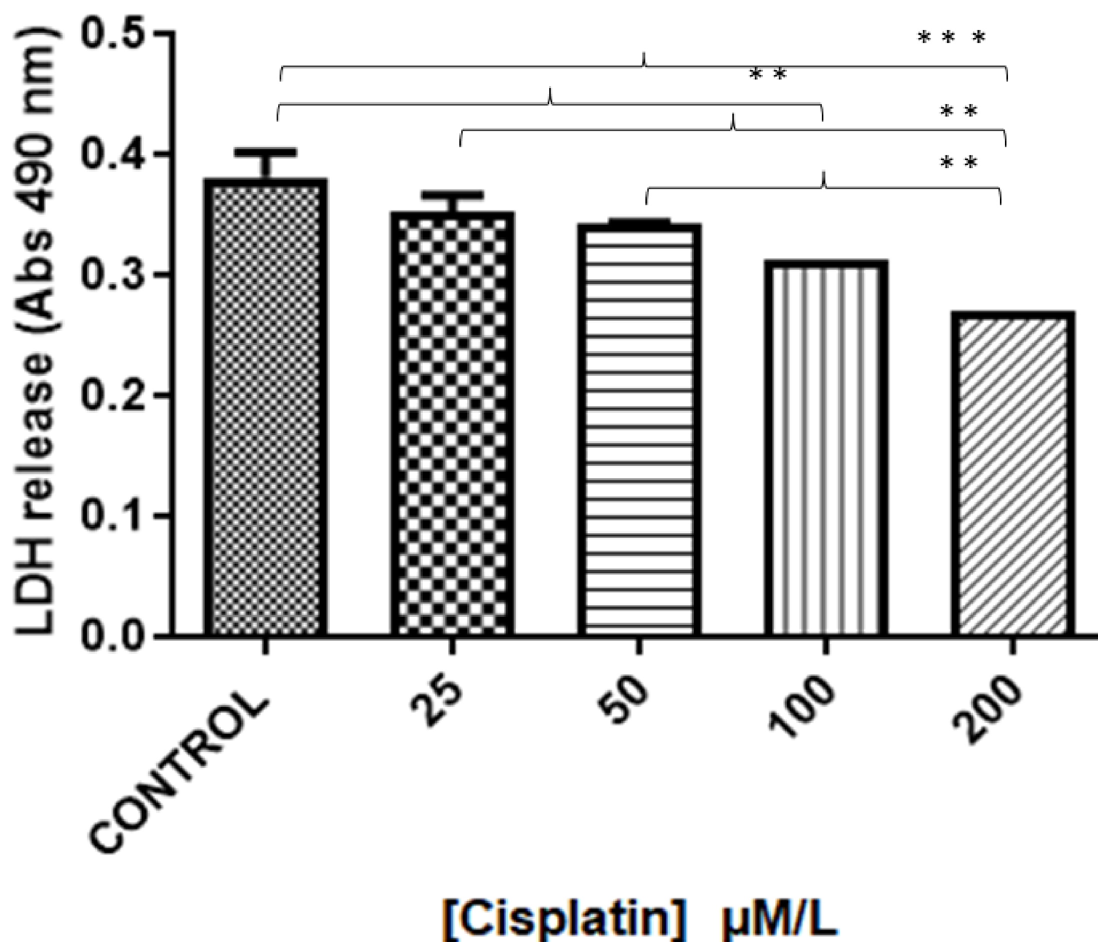

Figure 3. ALDH release in cultures of spheroids treated with different concentrations of CDDP. ANOVA port-hoc Tukey. ***p=0.001: **p=0.01: *p=0.05

CaCu spheroids *in situ* treated with 5-fu were shown to be resistant at the concentrations tested, which is in agreement with the results of previous studies on the Hs 766t cell line. In contrast, stage *IIB* exhibited EC$_{50}$ values lower than those previously reported. For PTX in CaCu, spheroid in *situ* EC$_{50}$ was not observed; in contrast, for the CaCu *IIB* sample, the EC$_{50}$ was lower than 25uM/L. The *in situ* CaCu spheroids treated with DXR were resistant to the tested concentrations, and EC$_{50}$ was not found in the assays. In contrast, spheroids from *IIB* samples showed EC$_{50}$ values lower than those reported previously (Table 1).

CDDP/PTX µM/L								
Cervix cancer *in situ*					Cervix cancer IIB			
0	50	150	250	350	0	50	150	250
100	1.5	2.0	1.71	2.0	100	1.5	1.5	1.71
200	0.99	1.66	1.66	1.66	200	1.5	1.5	1.66
300	1.75	1.75	NF	NS	300	1.5	1.75	NF
400	1.33	1.33	NF	NF	400	NF	NF	NF

5-FU/DXR μM/L								
Cervix cancer *in situ*					Cervix cancer IIB			
0	20	40	60	80	0	20	40	60
100	0.66	0.66	0.66	0.66	100	1.5	1.5	1.71
200	0.58	0.58	0.66	0.66	200	1.5	1.5	1.66
300	0.66	0.66	NF	NF	300	1.5	1.75	NF
400	0.66	0.83	NF	NF	400	NF	NF	NF

Table 1. Combinations of antineoplastics drugs. Inhibitory Fractional Concentration (IFC) or two combinations of antineoplastics drugs, the test was performed on spheroids formed from Cacu in situ and CacU IIB. CIF ≤ 0.5 synergism, CIF= 1 addition. ICF between 2 or 4 antagonisms, NF= IFC not found.

B. Assays to identify synergism-antagonism. CaCu *in situ*

Checkerboard tests were performed to determine synergism, antagonism, or additions when the two antineoplastic drugs with different mechanisms of action were combined. From the checkerboard assays, the Fractional Inhibitory Concentration Index (FCI) was determined, where values < 0.5 indicates synergism, 1 indicates addiction, and 2 and 4 indicate antagonism, depending on the literature. Next, we show the CFI of CDDP in combination with PTX, the assay was performed on spheroids formed from CaCu *in situ*, and no synergism was observed between these two drugs, at concentrations of 150/200 μM of CDPP-PTX respectively an indicator of 2 is observed, indicating antagonism. Similarly, we show the CFIs obtained from the combination of 5-fu in combination with DXR, which was performed on spheroids formed from CaCu *in situ*. The results showed that this combination was close to the synergism index, Table1.

C. Assays to identify synergism-antagonism. CaCu *IIB*

We demonstrated the CFI of CDDP in combination with PTX, which was performed on spheroids formed from CaCu *IIB*, and no synergism was observed between these two drugs. The indices obtained were added to all concentrations tested. The table below shows the CFI's of 5-fu in combination with DXR, and the assay was performed on spheroids formed from CaCu *IIB*. The results showed that the combination of both drugs at concentrations of 20/100 μM/L resulted in addition while increasing the concentration of DXR, but keeping that of 5-fu the index decreased (Table 1).

Discussion

In this study, we undertook the task of established spheroid cultures from cervix samples of CaCu, and our results indicate that human cervix tumor cells are viable for spheroid formation. However, we observed that when the cells were in the CaCu stage *in situ* the efficiency of the culture was 100%, while the samples in stage IIB were sometimes unable to aggregate and form spheroids (76% efficiency of culture formation). For cells in culture to aggregate and form spheres, they need culture plates with regions resistant to protein adsorption and cell adhesion. Cell adhesion and distribution are mainly dictated by integrins [19], and cell-cell binding is dictated by desmosomes. In general, this process takes a short period on unrestricted substrates (2D cultures). However, in 3D environments, cells often yield or proteolytically cleave physical scaffolds for spreading. In this case, cell spreading occurs over hours and, in some cases, days rather than minutes [20]. In our assays, some samples failed to aggregate, suggesting that there may be some change in the expression of cell-binding proteins and integrins as the cells were loosely suspended in the medium. These changes suggest that despite having samples at the same stage, when coming from different individuals, the expression of surface receptors directed by gene expression plays a fundamental role in cell behavior. Peñalver et al. reported that one of the most important characteristics of spheroids is their state of cell polarity, which is closely related to gene expression[12]. One of our specific objectives was to standardize cell-seeding density to obtain uniform spheroids and clear images. At a cell density of 2'000 cells per well, smooth rounded edges and good optical visualization of the entire spheroid diameter were obtained. In spheroid cultures, special attention must be paid to the seeding density and cell growth rate. These two parameters determine the size of the spheroid, which depends on the assay requirements. In this study, we examined the effects of four anticancer drugs used for chemotherapy of solid tumors, since we know that drug exposure time is an important factor when assessing viability and apoptosis[21], and we measured these two variables at four different times. The results indicate that the drug exposure time is not significant, and that in the 3D configuration, the cells are arranged in their own ECM, which complicates drug penetration into the spheroid [17]. This suggests that, in our spheroids, the ECM did not allow most of the drugs to penetrate the inner layers; therefore, we did not observe changes in viability with exposure time. However, in the same assay, DXR treatment significantly decreased the cell viability. After treatment, cell viability began to decrease and remained constant throughout the 120 h of treatment. This result was consistent with that of Gambar et al., who established that the use of DXR in murine models significantly decreased cell viability. Baek et al. investigated the cytotoxicity of DXR in 3D spheroids of basal alveolar epithelial adenocarcinoma (A549), cervix cancer (HeLa), neuroblastoma (SH-SY5Y), osteosarcoma (U2OS), and embryonic kidney (HEK293T) cell lines under real-time conditions (daily monitoring).

The results indicated that internalization by endocytosis was necessary for efficacy, as all cytotoxicities of all spheroids were visible after one day of treatment [22]. This is in agreement with our results, in which cell viability decreased after 24 h of treatment. Caspase activity in our spheroids was significantly decreased at 96 h of treatment, so for the following assays we used 96 h as a reference to measure changes in viability. We also observed that the drug had a significant effect on caspase activity; for CCDP, 5-fu, and PTX, a clear decrease was observed, while DXR maintained caspase activity as incubation time passed. These results suggest

that after a 96-hour treatment, the cells began to initiate mechanisms to evade caspase-mediated cell death. One mechanism by which tumor cells develop resistance to cytotoxic agents and radiation is related to resistance to apoptosis[(23)]. Apoptosis can be initiated by the activation of death receptors on the cell surface membranes (extrinsic pathway) or by a series of cellular events that occur mainly in the mitochondria (intrinsic pathway). We also evaluated the EC50s of the four antineoplastic drugs on spheroids and performed assays with CaCu *in situ* and CaCu IIB samples. To avoid discrepancies, we compared the EC50s obtained with those reported in the literature. The results indicated that EC_{50} changed depending on the stage of the sample. For *in situ* CaCu samples, the EC_{50} of CDDP and 5-fu were very similar to those reported in the literature; in contrast, for PTX and DXR, the reported EC_{50} was not observed to match the concentrations used in this study. Previous studies have shown that spheroids formed from endometrial cells are PTX-resistant [15]. However, in our study, CaCu IIB spheroids showed lower ECs_{50} than those reported previously; therefore, we cannot suggest a general resistance to PTX. In contrast, we observed that CDDP-treated spheroids showed a significant decrease in ALDH release, which was previously reported that ALDH is released into the culture medium after the loss of membrane integrity as a result of induced cytotoxicity. Cultures treated with the CDDP-PTX combination did not show significant cytotoxic effects. Therefore, we suggest that the chemoresistance of our spheroids to CDDP influences their responsiveness to the administered treatments. Particularly in cervix cancer, cell line cultures show higher levels of molecular markers related to Cancer Stem Cells (CSC) than monolayer cultures. A study by Sarabia Sanchez et al. attributed the resistance of spheroids of CaCu cell lines to CDDP in a high CSC population [18.] Despite this, the results obtained are important because we can observe the pharmacological response of each patient, which would greatly support the treatment plan in the search for a favorable response to chemotherapy. Currently, drug combination therapies for cancer are common in clinical practice; usually, one main drug is administered, and the other is administered as an adjuvant to treatment. Detecting synergism or antagonism in combination is important for predicting patient responses to treatment. In this study, we conducted trials of combinations of drugs with different mechanisms of action to investigate the first-line treatment responses. The results of the drug combinations revealed different behaviors at the tumor stage. For CaCu *in situ*, CDDP-PTX antagonism was found for CaCu in situ, which is interesting because combination drug therapies with taxanes (paclitaxel or docetaxel) and platinum analogs (carboplatin or cisplatin) are used as first-line cancer chemotherapy. In CaCu spheroids *in situ* the 5-fu-DXR combination showed values very close to synergism, whereas in CaCu IIB spheroids, we observed addition at different concentrations. Variations in responses are related to individual resistance, with each patient showing sensitivity to certain drugs and resistance to others. Recall that in the caspase activity assays, we noticed a decrease, suggesting that the cells put in place mechanisms to evade the action of caspases, and anti-apoptotic mechanisms such as Bcl-2 and Bcl-X, L could be involved.

Conclusions

Spheroid cultures can be formed from CaCu cells at different stages of the disease. The cell model used in this study may reflect future responses to chemotherapy, as results closer to the *in vivo* environment were obtained compared with monolayer culture. The combination of DXR/5-FU showed a better response in inhibiting cell viability than the conventional treatment (CDDP-PTX). This study showed that the patients responded differently depending on the treatment administered. This may be due to factors such as disease stage or interindividual variation. Therefore, personalized treatment has the potential to improve the response to chemotherapy in patients with UCB.

Declarations

Ethics approval and consent to participate

The samples were collected from the tumor bank of the Mexican National Institute of Cancer (http://www.incan.salud.gob.mx/. The samples provided informed consent and were authorized by the patient from whom biopsy was performed. The Protocol was approved by the Ethics and Research Committee of the Hospital (number 012/048/IMO / CB/20T).

Consent for publication

Not applicable

Availability of data and materials

The data analyzed in this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare not conflict of interest.

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Authors' contributions

AJTS performed analyses, data acquisition, and interpretation. DPPP drafted and revised the manuscript. JPU and FJPV interpreted data and drafted the manuscript. L.E.A.Q. performed human cancer cell analysis, interpretation, and drafted the manuscript. All authors approved the final version of the manuscript.

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