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Evaluation of *In Vitro* Efficacy of Vincristine and Cisplatin for Endometrial Cancer

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Abstract: Endometrial carcinoma is one of the most common gynaecological cancers affecting globally. The disease is managed excellently when confined to uterus only but fewer treatment choices are available for late line therapies including advanced and relapsed disease with drug resistance. Chemotherapy is considered as a cutting-edge treatment for such cases but requires more extensive investigation of different therapeutic drugs and their effects on cancer progression at genetic level. In this study, we have conducted an *in vitro* analysis of effect of two drugs namely, Cisplatin and Vincristine (alone and in combination) on human endometrial cancer patient serum exposed cell line RL95-2. The MTT assay was used to verify the vitality of treated cells and revealed the effectiveness of these drugs on cancer cells. The morphological changes in cell architecture were also observed with inverted microscope and SEM, showing altered shape and microvilli destruction of cancer cells following drug treatment. The gene expression levels of cancer cell lines after treatment met well with SEM results showing down regulation of COX-2, HIF-1 α , and VEGF genes which plays important role in disease progression. The promising anticancer effects of cisplatin and Vincristine as monotherapy and combination therapy were observed.

Keywords: Endometrial cancer, Combination therapy, *In vitro*, Cisplatin, Vincristine, Cancer cell line, COX-2, HIF-1 α , VEGF genes

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Introduction

Endometrial cancer (EC) is the sixth most common cancer in women diagnosed with cancer, and it is more common in high-income nations. In 2020, approximately 417,000 incident cases and 97,000 deaths were recorded from the disease worldwide

(Njoku *et al.*, 2022). Due to the increased prevalence of obesity and aging in the community, endometrial cancer is becoming more prevalent (Ha *et al.*, 2021; Seon *et al.*, 2023). By 2040, it is projected to be the third most prevailing cancer

and it is the fourth notable cause of cancer death among women (Eskander *et al.*, 2023). The condition is typically diagnosed after uterine bleeding or spotting that is irregular. According to previous studies, the five-year relative survival after diagnosis varies from approximately 85% in the United States to 73% in China, 71% in India, and 63% in Eastern Europe (Satishkumar *et al.*, 2022). In conformity with tumour histology and possible carcinogenesis, two types of EC are well known. Eighty per cent of instances of endometrial cancer are endometrioid EC, which is caused by excessive oestrogen exposure that, in the absence of progesterone's corrective effects, causes endometrial growth and endometrial hyperplasia. Because of their distinctive lack of differentiation, non-endometrioid EC (NEEC), which makes up 20% of instances of oestrogen-independent endometrial cancer, has a poorer prognosis (Van Weelden, 2019; Kuhn *et al.*, 2023).

When it comes to treating this terrible illness, hysterectomy is advised as the first line of treatment. Chemotherapy is thought to be beneficial for patients with high intermediate risk malignancies, nonetheless, based on pathologic characteristics and disease recurrence (Knisely, 2022; Restaino *et al.*, 2023). Current therapeutic findings have introduced the combination therapy (integration of different important drugs) in the chemotherapy of cancer. Combination therapy includes a fantastic phenomenon named as collateral sensitivity in which resistance of cancer cells to one drug is compensated by sensitivity to a second drug (Dalin *et al.*, 2022). One of the front-line platinum-based anticancer medications is cisplatin, which works by attaching the DNA to target cancerous cells. Transcription is inhibited and subsequent signal transduction in the cell is stopped as the medication binds to DNA. Regretfully, patients initially respond better to cisplatin-based medications; but, over time, the drug's clinical effectiveness is dramatically reduced when malignant tissue develops acquired or intrinsic resistance to it (Ramisetty *et al.*, 2023). Vinca alkaloid; Vincristine is found naturally in *Catharanthus roseus*. It is an antimitotic

medication that suppresses cancer cells by preventing the cell cycle through disruption of microtubule dynamics. This characteristic designates Vincristine as a helpful cytotoxic agent in cancer treatments. However, Vincristine has major adverse effects, primarily neurotoxicity, as a result of its transient biological impact (Skubnik *et al.*, 2020).

Despite all the chemotherapeutic barriers, cancer cells learn to flourish on the medication therapy itself, overcoming drug apprehension and continue to develop further. Hence, in spite of all medical progress, a wide range of chemotherapies fail to combat cancer. Identifying substitutes by which we can overcome resistance is crucial. Therefore, more advanced methods that do not depend exclusively on the cytotoxicity profile of a particular medication are needed to provide a more focused, effective, and improved kind of cancer treatment (Mokhtari *et al.*, 2017). It is noteworthy to observe that combination therapy, a kind of care that mixes two or more medicinal agents, offers hope for the treatment of cancer. The mixing of two anti-cancer drugs is known to enhance the treatment efficacy as compared to the monotherapy by targeting important pathways related to disease progression and dipping dose limiting single drug toxicity (Yap *et al.*, 2013; Jaaks *et al.*, 2022). Combination therapy inhibits the growth of tumours and may lessen medication resistance by stopping mitotically active cells, causing apoptosis as well as reducing its capacity to spread, the quantity of cancer stem cells is dropped, by triggering apoptosis (Vesaghhamedani *et al.*, 2017). Till now, the cornerstone of combination chemotherapy in cases of endometrial cancer is carboplatinum and paclitaxel drugs which are used in almost all endometrial carcinomas (Coleman *et al.*, 2023; Mirza *et al.*, 2023).

The efficacy of combination therapy including other drugs such as Vincristine and Cisplatin can be investigated. It can prove to be highly effective and can unlock doors to chemotherapeutic advancement in endometrial cancer. Little data is

available about the combined chemotherapeutic use of Cisplatin-Vincristine for endometrial cancer. This is thought to be of particular importance as treatment paradigms are changing quickly. Therefore, the aim of our research was to analyse the efficacy of Cisplatin and Vincristine alone and in combination on the cancer cell line RL95-2. Mostly drugs behave differently *in vitro* and in human endometrial micro environment. Therefore, we checked the effects of Cisplatin and Vincristine in human serum also.

Materials and Methods

Cell line procurement, maintenance and treatment:

The human EC cell line RL95-2 was purchased from the American Type Culture Collection (Rockville, MD, USA). A modified Eagle minimum essential medium (DMEM) from Dulbecco was used to cultivate the cells. It was augmented with 10% foetal bovine serum (FBS), 100 µg/ml streptomycin, 100 µg/ml penicillin, 0.001 mg/ml insulin, and Ham's F-12 media. The cells were then maintained in T-25 cm² culture flasks (Corning Inc. Corning, NY) at 37°C in a humidified incubator with 5% CO₂. As confluence reached 70–80%, cells were passaged and the medium was replaced every three days (Jang *et al.*, 2022). The cells were treated with 0.05% trypsin-EDTA (Thermo Fisher Scientific) in order to detach these for further study.

Ethics approval and consent to participate:

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional Research Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Institutional Ethics Committee of Postgraduate Institute of Medical, Education and Research (PGIMER), Chandigarh, India.

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The

patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

Human Serum (HS):

The ethical approval was obtained from the Institutional Ethics Committee of Postgraduate Institute of Medical, Education and Research (PGIMER), Chandigarh and written informed consent was obtained from all the patients enrolled in PGIMER, Chandigarh. The blood samples (5 ml) were collected in vials without anticoagulant from EC patients. Serum was isolated and stored in sterile tubes in aliquots at –80°C. The serum was used to expose the endometrial cancer cell line to provide human micro-environment simulating endometrial cancer patients.

Drug Formulation:

The drugs Cisplatin (Sigma-Aldrich Chemical Pvt Limited, Bangalore, India, Cat. #232120) and Vincristine (Cell Signalling Technology, Danvers; United States, Cat. #13043S) were purchased in the form of lyophilised powder. The stock solutions (10 mM) of both the drugs were prepared as per the instructions received from the manufacturer and stored at -20°C. Different concentrations of both the drugs were tested for the determination of IC₅₀ values using the stock solutions.

Calculation of IC₅₀ by MTT assay:

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, EMD Millipore Corp., USA] test was performed to ascertain the IC₅₀ or cell viability following treatment with varied dosages of the two drugs. The cells were subjected to 1.0, 2.0, and 4.0 µM doses of Vincristine and cisplatin for a duration of 48 h, as per prior research. The treated cells were housed in an incubator with 5% CO₂ that was humidified. At the conclusion of the experiment, the cells were washed with phosphate-buffered saline (PBS) and then MTT solution (0.5 mg/ml) was poured in individual well. Following a 3–4 h incubation

period, 100 µl of DMSO was added to dissolve the formazan crystals. Optical density was determined with a micro plate reader at 570 nm in wavelength (Agilent BioTek Epoch). Assays in triplicate were performed to obtain mean values for controls as well as treatment groups (Goyal *et al.*, 2021). The IC₅₀ value was measured using the formula given below:

$$\text{Cytotoxicity (\%)} = (a-b)/(c-b) \times 100$$

where a= Average absorbance of sample, b= Average absorbance of blank, c= Average absorbance of control.

Morphological Assessment of cells:

After treating a human serum exposed cancer cell line with IC₅₀ of Vincristine, Cisplatin, or both for 48 h, the morphological characteristics were examined using Scanning Electron Microscopy (SEM) and Inverted Light Microscopy. In order to perform SEM, cells were fixed in a glutaraldehyde-formaldehyde mixture for 2 h after being homogenized by trypsinization. The supernatant was disposed after these cells were centrifuged for 10 min at 1000 rpm. After that, the cell pellet was mixed with PBS solution and thoroughly shaken. The cells underwent another centrifugation for 10 min at 1300 rpm. A drop of cell suspension from each cell group was taken on stub with double adhesive tape. These samples were allowed to dry and extra fluid was soaked with filter paper. The prepared samples were viewed under Scanning Electron Microscope and micrographs were recorded (Nanou *et al.*, 2018).

RNA isolation and cDNA synthesis:

Using the TRIzol reagent (Ambion), RNA was manually extracted from the cells of the two main groups: Group I fetal bovine serum (FBS) and Group II human serum (HS). Both the groups were subdivided into Control, Vincristine, Cisplatin and combination (Vincristine + Cisplatin). By using a PCR machine (Prima-96TM thermal cycler, Himedia), 1 µg of RNA was reverse transcribed into cDNA using a first strand cDNA synthesis kit (Cat.#K1621, Thermo Scientific) as per the

instructions received from manufacturer. β- Actin was used as internal control.

Quantitative reverse transcription-polymerase chain reaction:

The expression patterns of all selected genes (COX-2, HIF-1α and VEGF) were studied in cells before and after treatment with the selected drugs. The Applied Biosystem was used to measure the transcription expression level of the aforementioned genes using the SYBR green master mix. The PCR reaction mixture (10 µl) was prepared by adding the following components (Table 1). Table 2 illustrates the primer sequences (designed for the experiment) and their melting temperatures (T_M). Melting peak analysis was performed to obtain the threshold cycle (CT) values and Livak's approach was used to examine the results.

Statistical analysis:

Experiments in triplicate were run. The statistical program GraphPad was used to assess the results. To assess variations amongst groups, one-way analysis of variance (ANOVA) was used, followed by post-hoc Tukey's multiple-comparison tests. The data re represented as mean ± SD.

Results

Cytotoxicity analysis:

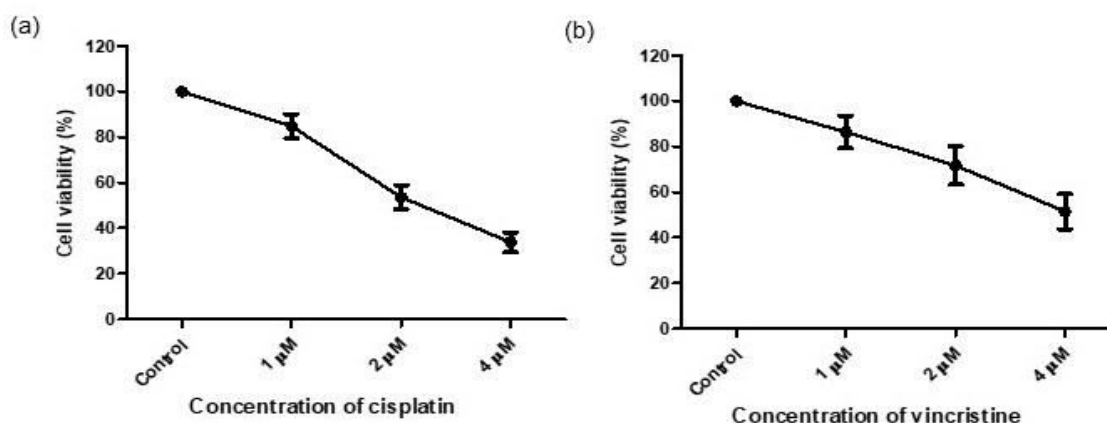
IC₅₀ values of Vincristine and Cisplatin on cancer cell lines were ascertained using the MTT test. Both Vincristine and Cisplatin had cytotoxic effects on cancer cells; however, the dose at which Cisplatin exhibited these effects was lower than that of Vincristine. After incubation for 48 h, the IC₅₀ value of Vincristine was found to be higher than that of Cisplatin. The IC₅₀ value of Vincristine came out to be 4 µM, whereas the IC₅₀ value of Cisplatin was found to be 2 µM. Vincristine at 4 µM concentration inhibited the proliferation of 53.84% RL95-2 cells when compared with control cells. Treatment with Cisplatin resulted in the inhibition of 53.62% cells at 2 µM concentration in contrast to control cells (Figs.1a, b). Based on these results, these two concentrations of

Table 1: Components of PCR reaction mixture

S. No.	Components	Volume
1.	SYBR Green PCR Master Mix	3 μ l
2.	Primers	1 μ l
3.	cDNA	1 μ l
4.	RNase free water	4.8 μ l
5.	High ROX (Kapa SYBR® Fast; Cat. #KK4600)	0.2 μ l

Table 2: Primer sequences used for different target genes

Gene	Sequence	T _m (°C)
β -Actin	F: GAATTGCTATGTGTCTGGGT R: CATCTTCAAACCTCCATGATG	57°C
COX-2	F: TCGACTGAACTGTAGAAGTCT R: CTTTCTCCGCAACAGGAGTA	58°C
HIF-1 α	F: GCAGCAACGACACAGAACT R: CTGCAGGGTCAGCACTACTT	57°C
VEGF	F: CCATTGTGGAGGCAGAGAAA R: AAGAGAGCAAGAGAGAGCAA	58°C

Fig. 1: Graphs showing 50% inhibitory concentration (IC₅₀) of (a) Cisplatin and (b) Vincristine in RL95-2 cells. Results are shown as mean $\pm$ SD.

Vincristine (4 μ M) and Cisplatin (2 μ M) were chosen to explore the effects on morphological and genetic expression changes.

Topographical studies:

The morphological changes of untreated and treated RL95-2 cells with Vincristine and Cisplatin at selected doses were studied. Following 48 h of drug treatment, light microscopic observations revealed that Vincristine and Cisplatin both reduced the density of treated cells grown in FBS

and caused a severe deformity in their shape. The untreated cells showed the normal cell morphology evident with intact cell membrane (Fig. 2a). Treated cells revealed the deformed shape and shrinkage after exposure to both the drugs alone and in combination. Exposure of both the drugs resulted in comparable changes in cell morphology (Figs. 2b, c). The cells treated with combination of both drugs resulted in slightly more density of cells in contrast to both the drugs alone. However, the cellular density in

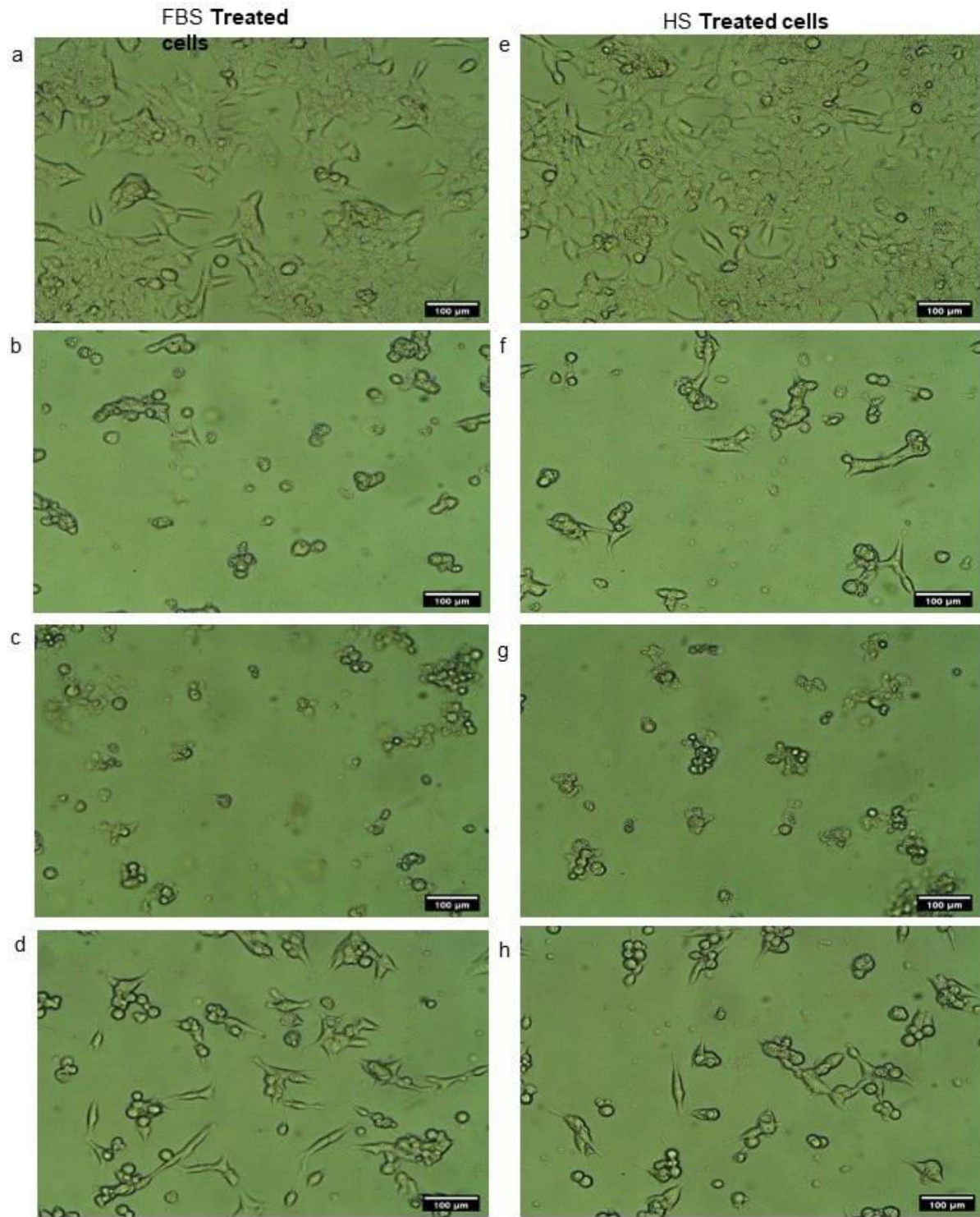


Fig. 2: Light microscopic observations of RL95-2 cells grown in (a-d) fetal bovine serum (FBS) and (e-h) human serum (HS) after 48 h of drug treatment: (a) control cells in FBS; (b) Vincristine treated cells in FBS; (c) cisplatin treated cells in FBS; (d) cells treated with combination of Vincristine and Cisplatin in FBS; (e) control cells in HS; (f) Vincristine treated cells in HS; (g) Cisplatin treated cells in HS and (h) cells treated with combination of Vincristine and Cisplatin in HS as observed.

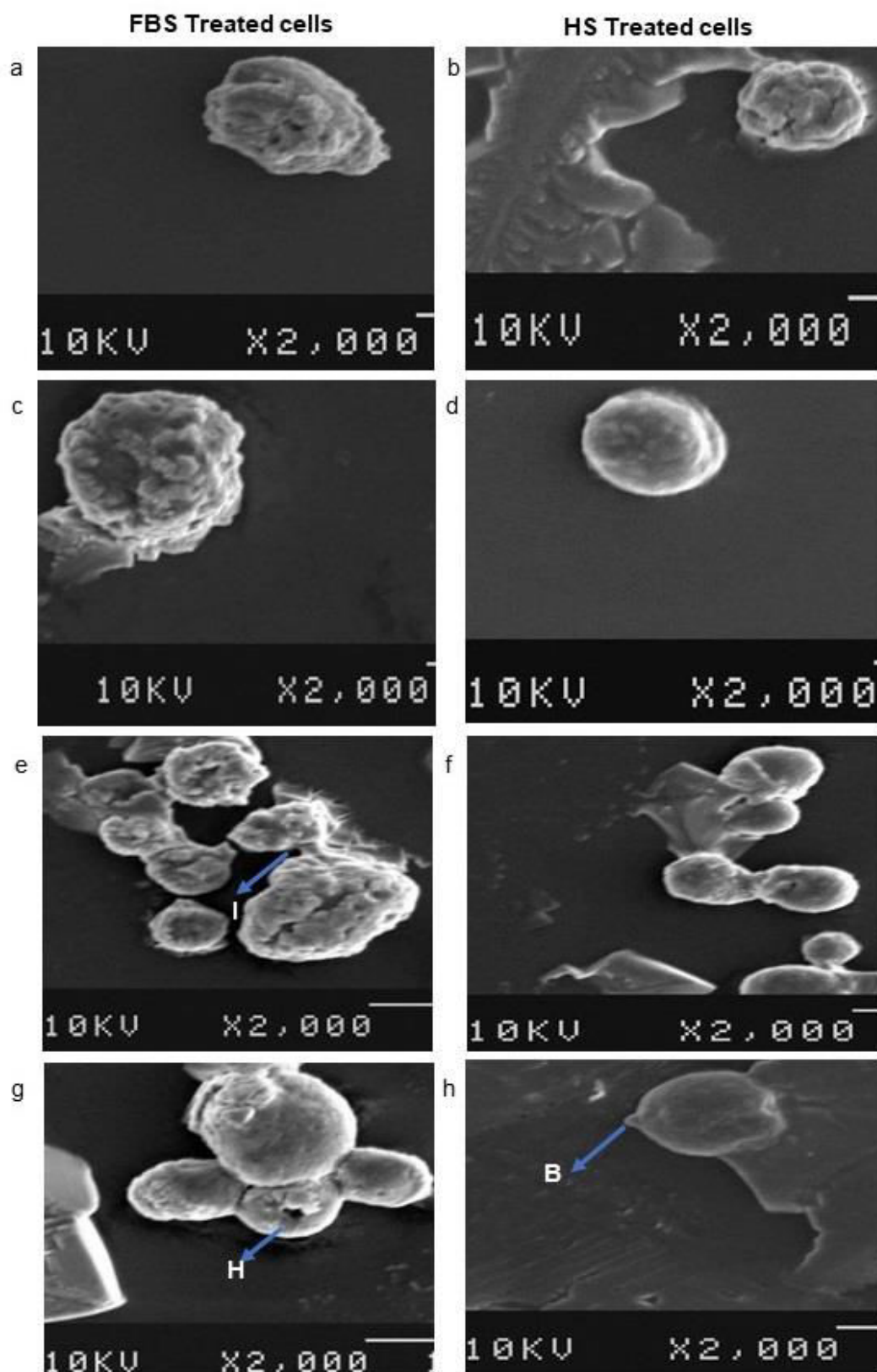


Fig. 3: SEM micrographs showing the morphological alterations in cancer cells before and after drug treatment: (a) control cells in FBS; (b) Vincristine treated cells in FBS; (c) Cisplatin treated cells in FBS; (d) cells treated with combination of Vincristine and Cisplatin in FBS; (e) control cells in HS; (f) Vincristine treated cells in HS; (g) Cisplatin treated cells in HS and (h) cells treated with combination of Vincristine and Cisplatin in HS as observed.

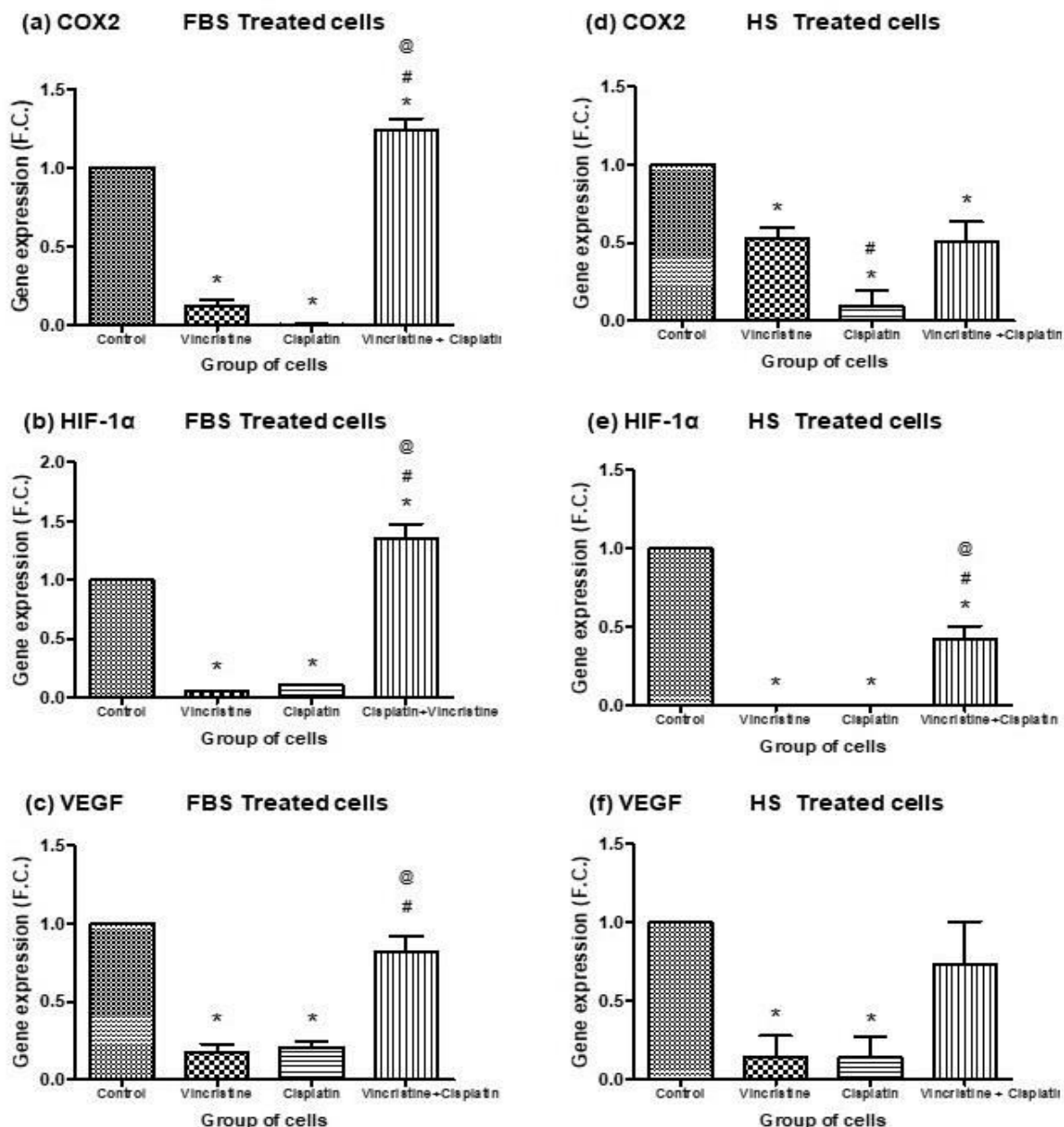


Fig. 4 (a-f): Gene expression levels of COX-2, HIF-1 α , and VEGF by qRT-PCR of untreated (Control) and treated cells (Cisplatin, Vincristine and Cisplatin + Vincristine) grown in fetal bovine serum (FBS) and human serum (HS). Values are presented as mean $\pm$ SD of three independent experiments. * $p < 0.05$ - significant difference as compared to the control, # $p < 0.05$ - significant difference between combination and Vincristine and Cisplatin alone exposed groups whereas @ $p < 0.05$ - significant difference between Cisplatin alone and combination group. Each experiment was performed in triplicate. One-way ANOVA followed by post-hoc Tukey's test.

combination group was significantly reduced when compared with group of control cells (Fig. 2d). In the group of cells grown in human serum, the cellular density was slightly more in the control group of cells (Fig. 2e). The observations on treated cells with the two drug alone and in combination were almost similar to that of FBS grown cells (Figs. 2f-h).

Moreover, the surface topography of the cells was examined using Scanning Electron Microscopy. The study examined the cellular structure of both treated and untreated cells cultured in human serum (HS) and foetal bovine serum (FBS). The untreated cells cultured in FBS and HS displayed microvilli rich cellular shape (Figs. 3a, e). Exposure of drugs at IC₅₀ of both the

drugs (Vincristine, Cisplatin and Vincristine + Cisplatin) for 48 h led to the altered cell morphology revealed in micrographs. The degree to which the drug therapy altered the cell's morphology and form varied depending on the kind of drug and treatment (alone or combination). The cells treated with Vincristine and Cisplatin alone attained a round or oval shape with significantly reduce in the number of microvilli (M) as compared to control. However, the cells grown in HS showed much reduction in microvilli as compared to cells grown in FBS (Figs. 3c-h). A prominent distortion of cell membrane (D) and cytoplasmic intrusion (I) were clearly visible in the Cisplatin treated cells as compared to Vincristine treated cells. When treated with the combination of Vincristine and Cisplatin, the cells showed distorted morphology with holes (H) and blebs (B). The crowding of microvilli seen in untreated cells lost in the treated cells and less crowding of microvilli on the cell surface was observed. Moreover, the cell surface became smoother in the cells grown in human serum as compared to FBS.

Gene expression analysis:

The relative gene expression of COX-2, HIF-1 α , and VEGF genes were assessed in the cancer cells grown in FBS and HS and treated with Cisplatin, Vincristine and combination of both drugs (selected dose). The expression levels of all the three assessed genes were found to be down regulated in the cells grown in FBS. The studied genes were significantly ($p<0.05$) down-regulated in the cells treated with Cisplatin and Vincristine alone when compared to the control cells. Comparing cells treated with a combination of Vincristine and Cisplatin to control cells, the down-regulation of VEGF was not statistically significant ($p>0.05$). When cells treated with a combination of Vincristine and Cisplatin were compared to control cells, they showed increased expression of HIF-1 α and COX-2. When Vincristine and Cisplatin were administered together, there was a significant ($p<0.05$) up-regulation of the examined genes compared to when these drugs

were administered separately. The expression levels of COX-2, HIF-1 α , and VEGF genes in cells treated with Vincristine and Cisplatin alone did not differ significantly (Figs. 4a-c).

The expression levels of COX-2, HIF-1 α , and VEGF genes in cells grown in HS and treated with Vincristine, Cisplatin, and their combination were significantly ($p<0.05$) down-regulated in comparison to control cells, with the exception of VEGF in cells treated with the combination. With the exception of COX-2, where there was a significant ($p<0.05$) difference in the expression levels of the studied genes between cells treated with Vincristine and Cisplatin alone, Cisplatin treatment led to further down-regulation of COX-2. Although the levels of gene expression of the three studied genes was found to be down-regulated in cells treated with combination of the two drugs in contrast to control cells but it was found to be up-regulated in cells treated with combination drugs when compared with cells exposed to Vincristine and Cisplatin alone. This upregulation of gene expression in cells exposed to combination drugs was significant ($p<0.05$) for HIF-1 α in comparison to both the drugs when exposed alone (Figs. 4d-f).

Discussion

Endometrial carcinoma is the sixth most widespread malignancy in women and the fifth most common cause of cancer death in the developed countries. The developing world is also not secluded from this fatal disease. Unfortunately, no improvement has been seen in the five-year, age-adjusted survival for endometrial carcinoma recently. Thus, this disease has become a major concern globally. Endometrial cancer management at initial phase of disease involves surgery followed by adjuvant treatment. Advanced or recurrent disease is treated majorly with chemotherapeutic agents depending upon the state of disease. Due to poor survival in the recurrent cases, there is an urgent need to seek novel drug therapies to improve disease supervision. Cisplatin causes cell damage by making DNA adjuvant and is a well-established

anticancer drug. Vincristine is a vinca alkaloid which acts through hampering microtubule assembly in the cell. This property of Vincristine can be explored for the treatment of cancer. Keeping this in mind, we have investigated the potential of these two drugs by exposing endometrial cancer cells to Cisplatin, Vincristine, and the combination of both the drugs.

Both Cisplatin and Vincristine have previously been reported to induce cell apoptosis factors. The cytotoxic effect of Cisplatin and Vincristine can be advocated on the basis of previous findings, HeLa cervical cancer cells treated with 100 nmol/L experienced significant cell death due to an early increase in lysosomal volume and lysosomal leakage, which was followed by the intrinsic apoptosis program being activated and microtubule assembly being disrupted (Groth-Pederson *et al.*, 2007). On the other hand, the majority of MCF-7 breast cancer cells treated with Vincristine showed resistance to apoptosis. As senescent cells that had undergone micronucleation, they managed to avoid mitotic arrest by increasing the volume and activity of their lysosomal compartment and adjusting to the spindle assembly checkpoint. Similarly, Lin *et al.* (2017) summarized the effect of Cisplatin (20 µg/ml) on endometrial cell line and demonstrated that Cisplatin treatment promoted cell autophagy in Ishikawa cell line which led to cell death.

The morphological analysis of the cancer cell line after treatment with Vincristine and Cisplatin using light microscopy revealed features characteristic of cell death as the cell shape was found to be changed from elliptical to rounded. The cell density was also reduced after treatment in contrast to normal control. SEM showed reduction in microvilli number, rounding-up of cell shape and blebbing following drug treatment. These results are in line with a previous study in which comparable alterations in the morphology of HCS-2 cells following treatment with elements of highly active antiretroviral therapy were observed (Xulu *et al.*, 2017). In our study, it was also noticed that Cisplatin showed more drastic

changes in cell texture as compared to Vincristine which may be due to more sensitivity of RL95-2 cells towards Cisplatin. Moreover, the ability of Cisplatin to disturb the cell cycle, reactive oxygen species production, DNA damage and autophagy contributed to its potential cytotoxic effect (Lin *et al.*, 2021). In our investigation, a group of cells treated with a combination of Vincristine and Cisplatin also showed distorted cells. Combination therapy targets several molecular pathways important for cell survival due to involvement of two different drugs thus improving treatment efficacy.

The genetic impact of Vincristine, Cisplatin, and combination drugs was also examined by measuring the expression levels of three key genes i.e., COX-2, HIF-1α, and VEGF. That is crucial for the advancement of cancer. The aforementioned genes may be essential to the development of endometrial cancer. Prior researches have indicated that COX-2 gene is unbecomingly induced and up-regulated in a number of malignancies and holds an important place in tumour cell biology by taking part in angiogenesis (St-German *et al.*, 2004). The cell's adaptive response to hypoxia is largely dependent on HIF-1α, another significant genetic marker. It also regulates important cellular functions such as glucose metabolism, erythropoiesis, apoptosis, vasomotor regulation, cell growth, heme metabolism and iron transport. Researchers recently examined the role of HIF-1α in endometrial cancer to assess the predictive and prognostic significance of HIF-1α protein expression. The positive expression of HIF-1α is majorly linked with poor prognosis and low survival in patients with cancer in endometrium (Zhu *et al.*, 2020). By encouraging angiogenesis, invasion, migration, and resistance to apoptosis, the VEGF signalling system in cancer cells is in charge of fostering carcinogenesis (Yeap *et al.*, 2021). In our study the expression level of all three genes was found to be downregulated in the cells exposed to Vincristine and Cisplatin which indicates the mechanism of action of these two drugs was dependent on the all three studied

genes. The down-regulation was more prominent in case of the cells exposed to Cisplatin and Vincristine alone whereas it was not upto that extent in the cells exposed to the combination of both the drugs. The inhibited down-regulation of the studied genes in cells treated with combination of the Cisplatin and Vincristine could be due to the antagonistic effects of the two drugs. *In vitro* antagonism has been reported earlier between Cisplatin and Vincristine on lung cancer cells (Lee *et al.*, 1989).

Conclusion

This study showed that Cisplatin and Vincristine monotherapy and combination therapy potentially affected the RL95-2 endometrial cancer cell line by inducing antiproliferative effect. The cytotoxic potential of monotherapy of Cisplatin and Vincristine showed promising results as compared to combination therapy involving both the drugs simultaneously. The drugs were capable of disrupting cell morphology and gene expression levels of COX-2, HIF-1 α and VEGF genes. These alterations pointed towards the powerful inducer of cell cytotoxicity which again confirmed the antitumorigenic effect of Cisplatin and Vincristine against endometrial cancer cells. Thus, it can be said that anti proliferative effects of Cisplatin and Vincristine are due to down regulation of cancer promoting gene of COX-2, HIF-1 α and VEGF. However, some other factors present in the human serum may be responsible for the discrepancy seen in the results obtained from FBS and HS exposed cells, which needs further investigation.

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