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Effect of HeLa Cell Density Towards Cisplatin Treatment

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Abstract

Cervical cancer is the fourth most prevalent cancer affecting women worldwide, especially those in low- and middle-income countries (LMICs). In treatment, cisplatin dosage is limited due to its adverse side effects. Studying the correlation between tumor size and cisplatin dosage is crucial to determine the right drug dose for cancer treatment. The purpose of this study was to investigate the anticancer effect of cisplatin on HeLa cells of different densities. Serially diluted HeLa cells with different seeding densities were treated with varying cisplatin concentrations (0µM-50µM), and their viability was assessed via MTS assay. From the findings, in comparison with non-treated HeLa cells, cisplatin caused significant reduction in cell viability by (1.80-10.80%) in 20000 cells, (5.25-15.25%) in 10000 cells, (5.95-63.15%) in 5000 cells, (8.95-67.15%) in 2500 cells, (16.20-83.00%) in 1250 cells, and (50.00-100.00%) in 625 cells correspondingly in a dose-dependent manner. An IC50 of 23.3µM, 19.8µM, 14.7µM, and 5.8µM was observed at 5000, 2500, 1250, and 625 densities respectively. Altogether, this study suggests that 5000 seeding density is the most suitable density to be used in cancer research as it mimics tumor cells' phenotype *in vitro* and produces more reliable results that is essential for *in vivo* application.

Keywords: HeLa; Cervical cancer; Cisplatin; Seeding density

Introduction

Cervical cancer is ranked as the fourth most prevalent cancer among women worldwide after breast, colorectal, and lung cancers (WHO, 2022) in which higher incidence and mortality rate is recorded in low- and middle-income countries (LMICs) than in well developed countries (Small *et al*., 2017). The introduction of formalized screening and HPV vaccination programs in highly developed countries have made it possible to reduce cervical cancer incidence and mortality by more than half (Cohen *et al*., 2019). Persistent infection of the cervix due to high-risk Human Papillomavirus (HPV) subtypes 16 and 18 accounts for almost all cervical cancers (Denny *et al*., 2015). With regular screening tests or pelvic examination, cervical cancer can be identified (Cohen *et al*., 2019). Early prognosis is much needed in order to select the best and suitable treatment as the stage and molecular subtypes differ between each patient. Although various treatments have been practiced over decades to save cervical cancer patients, many have also lost their lives due to inefficiency and recurrence or distant metastasis (Li *et al*., 2016).

Today, cisplatin is used widely as an approved chemotherapeutic agent to treat cervical cancer caused by various cell lines, such as HeLa and SiHa because of its ability to interfere with DNA repair mechanisms causing DNA damage, and inducing apoptosis in tumor cells (Dasari & Bernard Tchounwou, 2014). Even though cisplatin portrays a good reaction in chemotherapy, in some cases it results in cancer relapse and resistance towards treatment (Brown *et al*., 2019). To overcome this issue, cisplatin is administered at lower doses with other therapeutic drugs to maximize its efficiency in inducing cytotoxic effect in cancer cells (Dasari & Bernard Tchounwou, 2014; Yi *et al*., 2020).

Researchers have been captivated by the promising role of cisplatin in altering cancer cell mechanism, and thus are conducting further studies to improve its anti-tumorigenic properties while minimizing the side effects (Zhu *et al*., 2016; Zhang & Lu, 2021).

Despite the ability of cisplatin to induce significant anti-tumorigenic properties in various tumor cell including cervical cancer, there is always an issue with cisplatin dosage and its adverse side effects. Thus, it is crucial to study the correlation between the tumor size and cisplatin dosage. Therefore, in this study, the effect of cisplatin treatment on HeLa cells at different seeding densities were investigated.

Materials and methods

Reagents and cell culture. Cisplatin was purchased from Tocris Bioscience, and CellTiter 96® AQueous One Solution Assay was acquired from Promega. Cervical cancer cells (HeLa) was obtained from the Cancer Research Laboratory (Faculty of Science, Universiti Teknologi Malaysia) and was maintained in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12+Glutamax™) enriched with 10% Fetal Bovine Serum (FBS), and 1% antibiotic (100U/mL penicillin + 100µg/mL streptomycin) that were all purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The cells were incubated at 37°C in 5% CO^2 incubator.

HeLa cells of 20000 cells were seeded into specific wells in 96-well plates and were serially diluted to achieve desired seeding densities of 10000, 5000, 2500, 1250, and 625 cells. After 24 hours of incubation, the cells were treated with different cisplatin concentrations ranging from 0 to 50µM. After 24 hours, 20µL of MTS reagent was added into each well and the well plate was incubated for about 4 hours. Then, the absorbance at 570nm was measured using Elisa microplate reader.

Statistical analysis was carried out to compare and analyze the cytotoxic activity of varying cisplatin doses on different HeLa cell densities. Microsoft Excel was used to calculate the Student's t-test while GraphPad Prism 9 was used to generate graphs and find the IC50 values. Experiment was repeated two times and triplicate results were portrayed as mean ± SD.

Results and discussion

Different densities of HeLa cells were treated with varying concentrations of cisplatin. From the results, it was revealed that cisplatin induced significant reduction in HeLa cell viability in a dose-dependent manner but at different strength. The viability of the cells was assessed using CellTiter 96® AQueous One Solution Assay in which quantification of ATP was done in order to determine the number of viable cells in culture. The reading based on the colorimetric method is directly proportional to the number of viable cells.

Figure 1 (A) shows the cytotoxic effect of cisplatin on HeLa cells at 20000 density in a dose-dependent manner, ranging from 0µM (denoted as non-treated (NT)) to 50µM. As seen in figure 1 (A), the cisplatin showed non-significant reduction in HeLa cell viability at all doses. The viability only reduced by 1.80% when treated with 5µM cisplatin, followed by 4.00% at 10µM, 5.20% at 15µM, 6.10% at 20µM, 8.10% at 25µM, and 10.80% at 50µM when compared with non-treated (NT) HeLa cells containing 100% of viable cells. Figure 1 (B) displays the inhibitory effect of cisplatin at increasing concentrations on 10000 HeLa cells. As shown in figure 1 (B), all the cisplatin doses caused non-significant reduction in the viability of HeLa cells. When administered with 5µM cisplatin, only 5.25% inhibitory effect was observed, 8.50% at 10µM dose, 9.15% at 15µM dose, 11.10% at 20µM, 13.65% at 25µM, and 15.25% at the highest concentration, which is 50µM when compared with NT HeLa cells.

Figure 2 (A) displays the morphological changes in HeLa cells of 20000 density while figure 2 (B) shows the morphological changes of HeLa cells of 10000 density before and after treatment with cisplatin for 24 hours. As shown in figure 2 (A) and figure 2 (B), non-treated HeLa cells that served as control were adherent and exhibited epithelial-like morphology with intact plasma membrane. The cell-to-cell attachment was too high in a very compact space, and thus some of the cells appeared dead even before treatment as they could not obtain the needed nutrients to sustain their growth. Once cisplatin was administered at increasing doses, not much of a difference was observed in the viability because most of the cells were still alive. This finding suggests that when the cell density (20000 cells

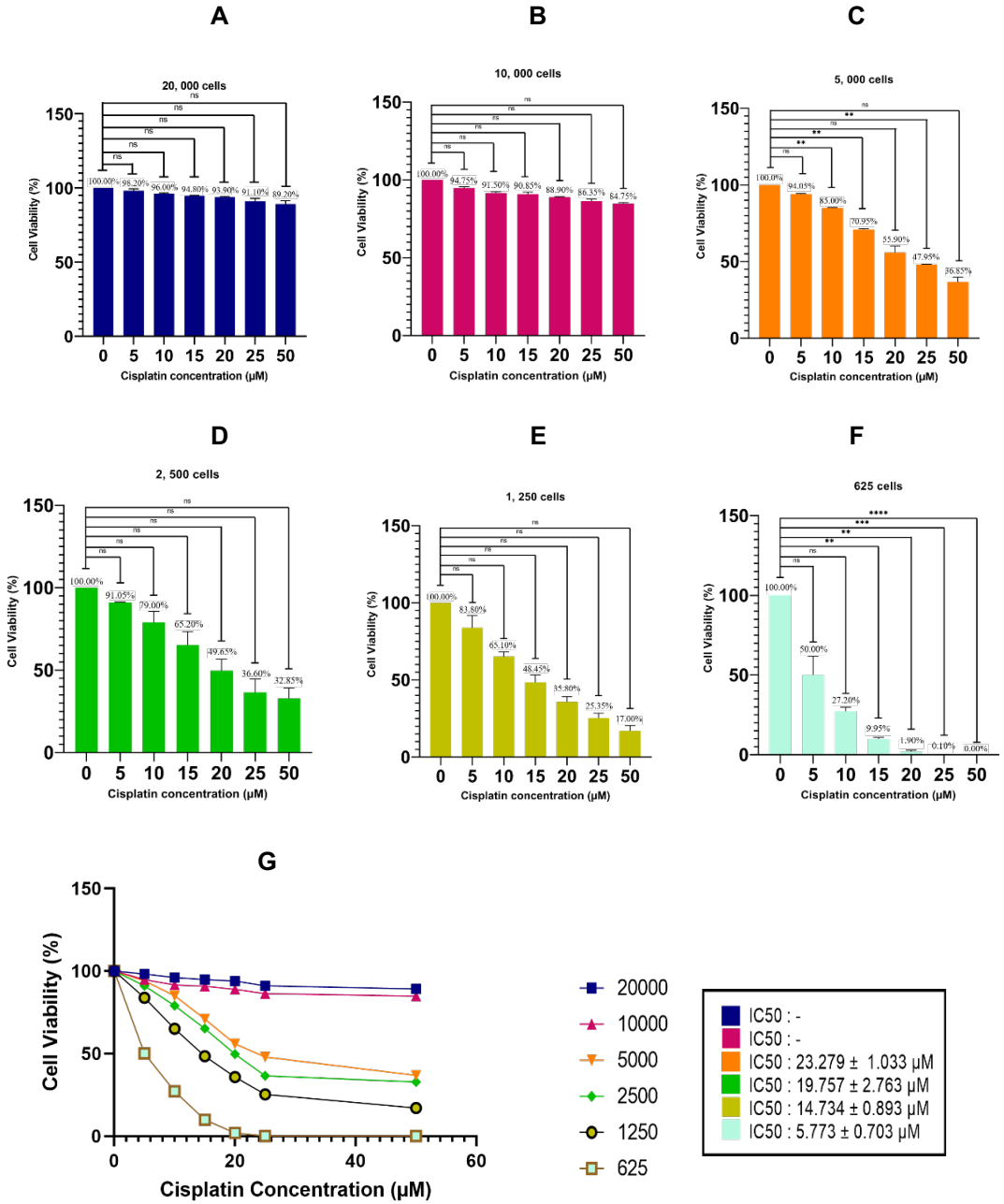

Figure 1 Cell viability assay of cisplatin on HeLa cells. (A) Effect of increasing cisplatin concentration (0, 5, 10, 15, 20, 25, 50 µM) on the viability of HeLa cells at 20000 cell density. (B) Effect of increasing cisplatin concentration (0, 5, 10, 15, 20, 25, 50 µM) on the viability of HeLa cells at 10000 cell density. (C) Effect of increasing cisplatin concentration (0, 5, 10, 15, 20, 25, 50 µM) on the viability of HeLa cells at 5000 cell density. (D) Effect of increasing cisplatin concentration (0, 5, 10, 15, 20, 25, 50 µM) on the viability of HeLa cells at 2500 cell density. (E) Effect of increasing cisplatin concentration (0, 5, 10, 15, 20, 25, 50 µM) on the viability of HeLa cells at 1250 cell density. (F) Effect of increasing cisplatinconcentration (0, 5, 10, 15, 20, 25, 50 µM) on the viability of HeLa cells at 625 cell density. (G) Overall comparison of cell viability assay after cisplatin treatment of different concentration on varying HeLa cell density. Experiments were conducted in triplicate and all data were presented as mean ± SD. Statistically significant values, *$p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ according to Student's t-test.

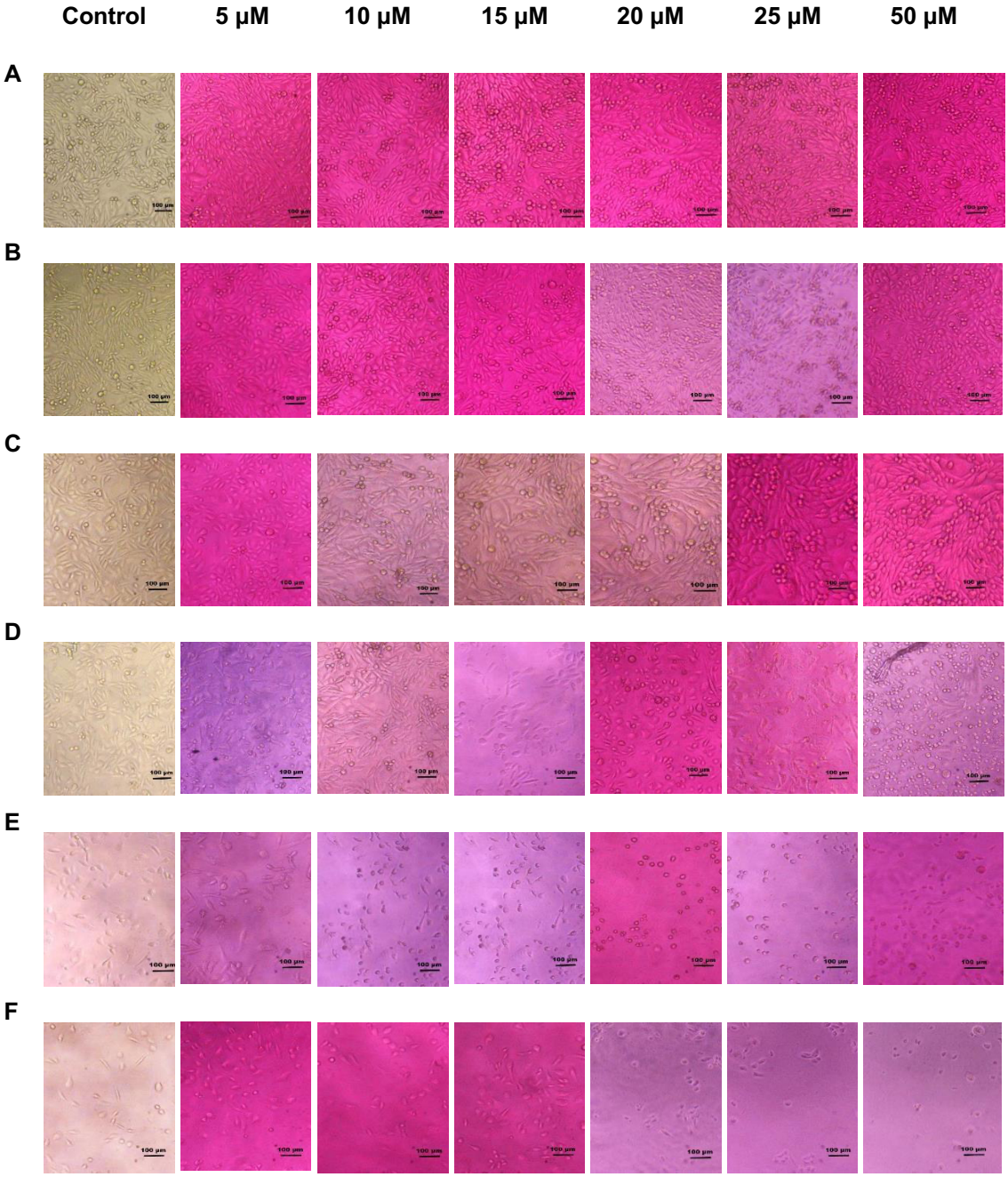

Figure 2 Microscopic images of HeLa cells before and after cisplatin treatment. (A) Morphological changes in HeLa cells of 20000 density induced by cisplatin at increasing doses (0 (NT), 5, 10, 15, 20, 25, 50 µM). (B) Morphological changes in HeLa cells of 10000 density induced by cisplatin at increasing doses (0 (NT), 5, 10, 15, 20, 25, 50 µM). (C) Morphological changes in HeLa cells of 5000 density induced by cisplatin at increasing doses (0 (NT), 5, 10, 15, 20, 25, 50 µM). (D) Morphological changes in HeLa cells of 2500 density induced by cisplatin at increasing doses (0 (NT), 5, 10, 15, 20, 25, 50 µM). (E) Morphological changes in HeLa cells of 1250 density induced by cisplatin at increasing doses (0 (NT), 5, 10, 15, 20, 25, 50 µM). (F) Morphological changes in HeLa cells of 625 density induced by cisplatin at increasing doses (0 (NT), 5, 10, 15, 20, 25, 50 µM). All the images were obtained using an inverted microscope at 10X magnification. (Scale bar: 100 µM)

& 10000 cells) is too high, lower cisplatin dose is not enough to exert a significant anticancer effect.

The reduced sensitivity of HeLa cells to cisplatin is associated with decreased intracellular uptake of platinum compound due to cisplatin resistance (Zhu *et al*., 2016). Limited cisplatin uptake in the cells have restricted the formation of cisplatin-DNA adduct and is insufficient to trigger apoptosis via DNA damage (Zhu *et al*., 2016). Research by Wieringa *et al* (2016) suggested that the tumor cells respond to DNA damage by activating the DNA damage response (DDR) in which DDR-proficient tumor cells were able to repair therapy-induced DNA damage and were more resistant to therapy than tumor cells with inactivated DDR. Also, several studies suggested that the downregulation of copper transporter 1 (CTR1), a major copper influx transporter in human cells was responsible for decreased cisplatin uptake in cisplatin-resistant HeLa cells (Zhu *et al*., 2016; Kilari *et al*., 2016). Furthermore, the cisplatin was not able to cause death in much cells due to HeLa cells' high proliferative and metabolic activity. At high seeding density, the cell number doubled after 24 hours of incubation, consequently leading to over confluent wells (Ghasemi *et al*., 2021). Alterations in the nature of intercellular signaling and cells' metabolic activity affected the drug dosage required to kill the tumor cells and also the level of formazan produced, thus leading to inaccurate absorbance reading (Ghasemi *et al*., 2021). From this finding, it could be presumed that reduced cytotoxic effect in HeLa cells may be caused by cisplatin resistance and constantly changing cell population.

Figure 1 (C) shows the inhibitory effect of cisplatin in 5000 HeLa cell density after 24 hours. From the figure, it can be seen that cisplatin induced cytotoxic effect by 5.95% at 5μM, 15.00% ($p < 0.01$) at 10μM, 29.05% ($p < 0.01$) at 15μM, 44.10% at 20μM, 52.05% ($p < 0.01$) at 25μM, and 63.15% at 50μM, with IC50 of 23.3μM in comparison with NT HeLa cells (Figure 1 (G)). In figure 2 (C), it can be observed that the morphology of cisplatin-treated HeLa cells at 5000 density showed epithelial-like shape in monolayer with less space in between attached cells. As the cisplatin dosage got higher, significant reduction was noticed in the viability of cells where more cell shrinkage and membrane blebbing occurred. Meanwhile figure 1 (D) represents the inhibition of cell viability by cisplatin treatment on 2500 cell density. Figure 1 (D) revealed that at 5μM dose, cisplatin was able to reduce the viability of HeLa cells by 8.95% when compared with NT cells, 21.00% at 10μM dose, 34.80% at 15μM, 50.35% at 20μM, 63.40% at 25μM, and 67.15% at 50μM in a non-significant manner. The IC50 of cisplatin at 2500 cell density was recorded at 19.8μM (Figure 1 (G)). From figure 2 (D), the morphological changes in HeLa cells of 2500 density can be seen. In control, the HeLa cells represented a monolayer with some space spotted in between cells. Most of the cells appeared dead when higher concentration of cisplatin (20, 25, 50μM) was administered. This result shows that at optimum seeding density (5000 & 2500), cisplatin was able to induce significant cytotoxic effect in HeLa cells.

Research by Luong *et al* (2016) suggested that at increasing dosage, higher amount of cisplatin was able to enter the tumor cells via CTR1 and formed cisplatin-DNA adducts, triggering DNA destruction that caused suppression of DNA replication and transcription. Cellular signal transduction proteins such as p53, p73, and amino-terminal kinase JUN that control cell growth and differentiation were involved in the recognition of the adducts and in the DNA repair mechanism (Tsvetkova & Ivanova, 2022). Activation of DDR in an effort to repair the damaged DNA caused cell death as cisplatin resulted in cell cycle arrest at G1, S, or G2 phase (Aldossary, 2019). Cisplatin was said to be capable of inducing reactive oxygen species to trigger cell death in tumor cells via extrinsic and intrinsic pathway (Dasari & Tchounwou, 2014). Cisplatin was believed to be involved in the formation of oxidative stress in mitochondrion of cancer cells which can result in loss of mitochondrial protein sulfhydryl group, calcium uptake inhibition, and reduction of mitochondrial membrane potential (Dasari & Tchounwou, 2014). From this study, it can be deduced that cisplatin induced significant anticancer effect in HeLa cells of medium density ranging from 5000 to 2500 cells.

In Figure 1 (E), non-significant reduction in the viability of HeLa cells at 1250 cell density was observed after 24 hours of cisplatin treatment. When compared with NT HeLa cells, cisplatin dose of 5μM was able to exhibit 16.20% inhibitory effect in 1250 HeLa cells, followed by 34.90% at 10μM, 51.55% at 15μM, 64.20% at 20μM, 74.65% at 25μM, and 83.00% at 50μM. The IC50 value of cisplatin in 1250 cell density was recorded at 14.7μM (Figure 1 (G)). On the other hand, in figure 1 (F), when the cisplatin concentration was 5μM, and 10μM, non-significant reduction was observed in the viability of

625 HeLa cells where the inhibitory effect was 50.00% and 72.80% respectively. However, cisplatin showed significant cytotoxic effect in HeLa cell viability by 90.05% ($p < 0.01$) at 15μM concentration, 98.10% ($p < 0.01$) at 20μM, 99.90% ($p < 0.001$) at 25μM, and 100.00% ($p < 0.0001$) at highest concentration of 50μM, with IC50 of 5.8μM (Figure 1 (G)).

Figure 2 (E) shows the changes in morphology of HeLa cells at 1250 density while figure 2 (F) depicts the changes in 625 HeLa cells pre- and post-cisplatin treatment. From both the figures, it can be observed that the control HeLa cells were adherent and showed epithelial-like morphology before treatment with very little cell-to-cell interaction. Very large space was spotted in between the cells, and thus affecting the proliferation, self-renewal property, and adhesion properties of the tumor cells (Yassin *et al*., 2015). The administration of a very little dosage of cisplatin have caused a major decrease in the density of live cells where almost all cells appeared apoptotic-like with membrane blebbing. This result indicates that at lower cell densities (1250 cells & 625 cells), usage of less amount of cisplatin have caused a significant reduction in the tumor cell density.

Cancer cells interact with each other in the tumor microenvironment to generate signals for survival and lineage (Brady-Kalnay, 2012). Research by Dominiak *et al* (2020) suggested that when cells were seeded at too low density, the cell-to-cell interaction and communication was limited. Thus, the tumor cells showed slow growth and eventually died even before treatment. When cisplatin was administered at lower dosage, the compound reacted with HeLa cells that were still alive, causing more cellular death. From this result, it can be summarized that lower cell densities are not suitable to be used in cancer related studies because increased cell-to-cell-interaction is crucial for the development of stem cells niche to determine their shape and also their function (Abdal Dayem *et al*., 2018).

Hence, from this study, it can be concluded that cell seeding density of 5000 is the most suitable size to be utilized in cancer research to determine the most effective cisplatin dose for cancer treatment while inducing less side effects. The IC50 concentration achieved by cisplatin in this seeding density is in the range approved for chemotherapy, which is in between 20mg/m2 to 100mg/m2 in 21 to 28-day cycles (Gold & Raja, 2021). Lower cisplatin dosage was able to cease the proliferation of tumor cells via sustained activation of the DNA damage checkpoint (Luong et al., 2016). However, the cells may re-enter cell cycle progression after acquiring additional changes, possibly causing cancer relapse even though the cell fate halts the growth of tumor cells (Luong et al., 2016). On the other hand, higher dosage of cisplatin is associated with severe side effects such as nephrotoxicity, hepatotoxicity, and neurotoxicity. Thus, to improve the efficacy of cisplatin in inducing significant anticancer effect in HeLa cells with minimal side effects, addition of secondary anticancer drug is important to ensure overall improvement in cervical cancer treatment.

Conclusion

In conclusion, cisplatin of 15 to 25μM is the most effective dose to exert an anti-cancer effect on HeLa cells of 5000 density. To treat higher cell densities (20000 & 10000 cells), lower concentration of cisplatin is not suitable as the amount of drug uptake will not be enough to induce significant inhibitory effect in all cells. The usage of high dose cisplatin increases the anti-cancer effect along with the side effects of chemotherapy. On the other hand, lower cell densities (1250 & 625 cells) are not suitable for cancer cell study because the cell-to-cell attachment is too low to support the growth of the cells, thus leading to increased cell death even before treatment. Hence, the exact drug concentration needed to induce anti-tumorigenic effect can not be studied.

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