# Association Between Dose and Duration of Cisplatin Exposure with Cytotoxicity Effect on Nasopharyngeal Carcinoma Stem Cell

*by* Achmad Chusnu Romdhoni

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ORIGINAL ARTICLE

# Association Between Dose and Duration of Cisplatin Exposure with Cytotoxicity Effect on Nasopharyngeal Carcinoma Stem Cell

**Laksmi Kusuma Wardhani[1] · Widodo Ario Kentjono[1] · Achmad Chusnu Romdhoni[1]**

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**Abstract** Nasopharyngeal carcinoma (NPC) is ranked 6th of malignant tumors in Indonesia. To analyze the correlation of dose and duration of cisplatin exposure with cytotoxic effects on nasopharyngeal carcinoma stem cells. The biopsy NPC tissue was cultured and processed to obtain NPC stem cells to be treated with cisplatin different doses and durations (24 and 48 h). The number of dead cells after exposure will be calculated using a hemocytometer. Death stem cell density of NPC at exposure of 2 µg/ml cisplatin dose was 81.37%, while the smallest death cell density a dose of 0.05 µg/ml after a 72-h observation was 21.3%. The coefficient correlation 0.827 and value $p = 0.000$ ($p < 0.05$). The analysis of the correlation between cisplatin exposure duration and death cell was also significant with the correlation coefficient − 0.357 and the value $p = 0.001$ ($p < 0.05$). There was a correlation between the increased dose of cisplatin with the cytotoxicity effects on NPC stem cell.

**Keywords** Nasopharyngeal carcinoma (NPC) · Dose · Duration of exposure · Cisplatin · Cytotoxicity

## Introduction

The incidence of Nasopharyngeal Carcinoma (NPC) in Indonesia is 6.2/100,000 inhabitants every year. NPC is ranked 6th of malignant tumors in humans after malignant tumors of the cervix, liver, breast, lung, and skin [1]. According to research both national and international, it is reported that most of NPC patients (80%) come in advanced stages III and IV [2]. During the last five decades, NPC handling is mainly in various forms of chemotherapy and radiation therapy. The damage effect caused by chemotherapy is called cytotoxicity, that most chemotherapy drugs work by interfering cell mitosis and primarily targeting cells with high cleavage rates [3].

Cisplatin is an effective chemotherapy, but side effects and resistance problems are two major constraints limiting its application [4]. The biochemical mechanism of cisplatin cytotoxicity includes the correlation between DNA and non-DNA targets which will induce death cell through apoptosis, necrosis or both [5]. The cytotoxicity indicator used is EC50 (effective concentration 50) which is the dose/concentration of certain compounds that needed to produce a cytotoxic effect of 50% death cell in cell culture in vitro. This indicator is often used as a benchmark of eukaryotic cell cytotoxicity in culture [3].

Post-chemotherapy evaluation of NPC encountered that the cancer cell does not give the best response that about 60–70% shows partial response and 15–25% unresponded [6, 7]. The failure is the presupposition that caused by a resistance factor that is a major complication of cancer chemotherapy and responsible for the failure of it in the treatment of cancer patients. Several theories suggest the cause of resistance is the presence of resistant cancer stem cells (RCSC) to chemo nor radiotherapy. It cause their special biological intrinsic characteristics of self-renewal and the production of different new cells to form tumors [8].

Research that correlated the role of resistant cancer stem cells to cisplatin and the progression of many malignancies were included in ca mammae studies was reported that the

✉ Widodo Ario Kentjono
widodo.arioken@gmail.com

[1] Department of Otorhinolaryngology, Faculty of Medicine Universitas Airlangga - Dr. Soetomo General Hospital, Surabaya, Indonesia

presence of tumor cells were expressing the normal tumorigenic stem cell characteristics by 5.9% of the tumor cell population. The population of these cells significantly increased to an average of 8.8% in primary transplants that only responded partially to cisplatin, while in secondary tumor transplants, the population increased to 22.8% [9]. The mechanism of NPC cell resistance against cisplatin chemotherapy correlated with the presence of cancer stem cell that has not been demonstrated yet. The mechanism related to the presence of NPC stem cells is not known well. The first step to understand it is to answer the question of whether cisplatin is capable of producing cytotoxic effects on NPC stem cells. The dosage and duration of exposure required to eliminate NPC stem cells are also unknown because of the different cytotoxicity doses for each cell line [10–13].

In vitro research on the effect of cisplatin on DNA suggests that cisplatin toxicity is affected by dose and time/dose also time-dependent [10, 14]. The previous research mentioned the concentration of certain cisplatin was needed to kill 90% of cancer cells [15]. While the other studies reported that 24-h cisplatin exposure was significantly much more cytotoxic than the first hour duration of exposure [16]. Based on these descriptions, this study was conducted to reveal the cytotoxic effects of cisplatin chemotherapy drugs on NPC stem cells and the dose also duration that affect them. This approach useful for understanding how NPC stem cells process cisplatin exposure and determining the effect of the dose also the duration of cisplatin exposure resulting in cytotoxic effects on NPC stem cells.

## Materials and Methods

True experimental laboratory in vitro with the factorial design was used in this study. Biopsy specimens from a patient suspected of NPC were taken in sufficient quantities, and some were sent to the Anatomical Pathology Installation Dr. Soetomo General Hospital in formalin solution. Nasopharyngeal carcinoma stem cell culture divided into two plates.

After the culture procedure, stem cells nasopharynx carcinoma confirmed with CD44 + staining that divided into two plates of each containing 24 well. Each well is filled with 100,000 stem cells of nasopharyngeal carcinoma. With randomization, the three wells were each treated with cisplatin that according to the dose 0.05; 0.1; 0.2; 0.4; 0.8; 1, and 2 µg (including 3 wells which were controls, without the addition of cisplatin treatment). One plate was incubated for 24 h and another plate for 48 h, after 24 h exposure, NPC stem cells in one plate were cleared of cisplatin and observed at 24, 48, and 72 h before calculation. The same procedure for 48 h of cisplatin exposure was performed in a similar method. Performed the cell counting procedures and cell cytotoxicity determination (determine the total cell count in the suspension of the original solution, percentage of dead cells compared to the total cell counts in one cell suspension) as follows: (1) prepared dry hemocytometer with slip of glass cover; (2) note the volume of cell suspension in milliliters; (3) cell suspension mixed thoroughly with serological sterile pipette, transferred 100 µl (0.1 ml) cell suspension to a 24-well plate using a 1 ml sterile serological pipette; (4) added 100 µl (0.1 ml) (same volume) of trypan blue by 0.4% to cell sample using serologic pipette 1 ml, then mixed to 100 µl and reversed; (5) transferred 20 µl of the mixed cell into one or two chambers of hemocytometer using R-20 pipette. Let the chamber filled with more capillary mechanisms; (6) using a 10 × objective lens, the lines within the chamber were focused; (7) calculated number of dead cells (colored blue) in 4 square side one chamber; (8) the number of death cells (blue) in 4 angles within a chamber will be calculated. Cells located within the line must be counted only if the cell touches the upper and left border of each corner of the box [9]. From the results it was calculated the total number of cells in the original solution by using the formula Total cell/ml × 2 × $10^3$ and divided by 4 [10].

The density of the dying cell from the original mixture will be calculated according to the percentage of total number of dead NPC stem cells divided by total number of NPC stem cells (dead and alive cells).

## Results

The data shows the number of cells experiencing mortality at 24 h cisplatin exposure and observation periods at 24, 48 and 72 h. The number of cells that died after cisplatin administration increased along with increasing the doses (compared with controls). The pattern increase occurs with a little fluctuation were the decrease in the dose of 0.4 µg at 24 and 48 h of observation then increase again. At 72 h observation the decrease occurred at a dose of 0.8 µg and then the number of dead cells increased (Table 1).

Death stem cell density of NPC mostly in the 24 h cisplatin exposure was obtained at 2 µg/ml with the post-observation time after exposure was 81.37%, while the smallest death cell density at 0.05 µg/ml dose calculated after 72 h observation was 21.3% (Fig. 1). Figure 1 shows that there is a tendency to increase in death cell patterns by increasing doses. The proportion of dead cells was relatively high after post-24 h observation, while post-observation 48 and 72 h of the relatively close coincident pattern but not as high as post-24 h observation.

**Table 1** Number and density of dead cells in a post-exposure solution of 24-h cisplatin

| Concentration (µg/ml) | Observation 24-h | | | Observation 48-h | | | Observation 72-h | | |
|---|---|---|---|---|---|---|---|---|---|
| | Live | Dead | Death cell density (%) | Live | Dead | Death cell density (%) | Live | Dead | Death cell density (%) |
| 0.05 | 43,500 | 23,000 | 34.58 | 81,000 | 27,000 | 25.00 | 90,500 | 24,500 | 21.30 |
| 0.1 | 55,000 | 30,500 | 35.67 | 67,000 | 34,500 | 33.99 | 89,000 | 31,500 | 26.14 |
| 0.2 | 58,500 | 42,500 | 42.07 | 95,000 | 45,000 | 32.14 | 96,500 | 40,500 | 29.56 |
| 0.4 | 54,500 | 38,500 | 41.39 | 68,500 | 41,000 | 37.44 | 89,000 | 44,000 | 33.08 |
| 0.8 | 42,000 | 40,000 | 48.78 | 82,500 | 49,000 | 37.26 | 59,500 | 41,000 | 40.79 |
| 1 | 16,000 | 30,000 | 65.21 | 64,500 | 47,000 | 42.15 | 57,500 | 47,500 | 45.23 |
| 2 | 9500 | 41,500 | 81.37 | 53,500 | 60,500 | 53.07 | 32,500 | 39,500 | 54.86 |
| Control | 70,000 | 20,000 | 22.22 | 117,000 | 15,000 | 11.36 | 98,500 | 13,500 | 12.05 |

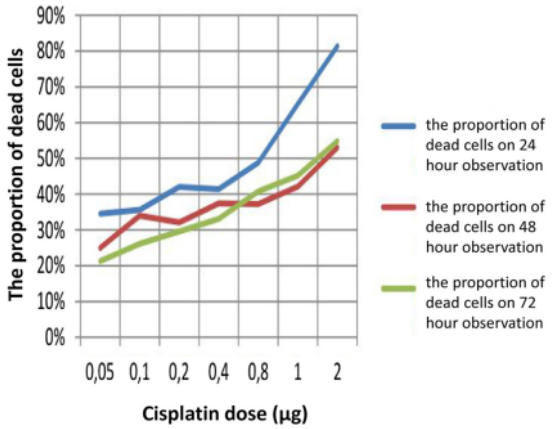


**Fig. 1** Graph of proportional correlation of KNF stem cell death on 24-h cisplatin exposure and different observation duration

Table 2 shows a similar pattern to 24-h cisplatin exposure that is an increase in dose-dependent death cell, there was a slight decrease in the dose of 0.1 µg, then increased again. On a 48-h observation, death cell was high at a dose of 0.05 µg, dropping at 0.1 µg, then just increasing again at 0.4 µg. On a 72-h observation, death cell dropped at a dose of 0.1 µg then increased at a dose of 0.2 µg. Density stem cells of NPC mostly at 48 h cisplatin exposure was obtained at 2 µg/ml cisplatin dose exposure with a post-observation time at 72 h after exposure was 51.32%, while the smallest death cell density at 0.05 µg/ml calculated on the 24-h observation was 8.45% (Fig. 2).

Figure 2 shows that death cell density after 48 h of cisplatin exposure tends to increase according to the observation period. The density of death cell at 48 h of observation was relatively higher than 24 h, while after 72 h observation showed the highest density. From statistical analysis with multiple regression correlation tests between the density of death cell (proportion of dead cells) and cisplatin dose was obtained the correlation coefficient 0.827 and $p = 0.000$. Figures 1 and 2 show tendency pattern of increased death cell with higher cisplatin doses. Both of these showed that there was a significant correlation between the increased dose of cisplatin and

**Table 2** Number and density of dead cells in the original solution of post-exposure to cisplatin 48 h

| Concentration (µg/ml) | Observation 24-h | | | Observation 48-h | | | Observation 72-h | | |
|---|---|---|---|---|---|---|---|---|---|
| | Live | Dead | Death cell density (%) | Live | Dead | Death cell density (%) | Live | Dead | Death cell density (%) |
| 0.05 | 92,000 | 8500 | 8.45 | 70,000 | 17,500 | 20 | 66,000 | 21,500 | 24.57 |
| 0.1 | 85,000 | 14,500 | 14.5 | 63,500 | 13,500 | 17.53 | 59,000 | 15,000 | 20.27 |
| 0.2 | 73,000 | 14,000 | 16.09 | 52,000 | 9500 | 15.44 | 47,000 | 16,500 | 25.98 |
| 0.4 | 91,500 | 13,000 | 12.44 | 46,500 | 12,500 | 21.18 | 41,000 | 17,000 | 29.31 |
| 0.8 | 44,500 | 12,500 | 21.92 | 53,500 | 16,500 | 23.57 | 33,500 | 17,500 | 34.31 |
| 1 | 35,000 | 16,500 | 32.03 | 46,000 | 24,000 | 34.28 | 32,500 | 21,500 | 39.81 |
| 2 | 34,500 | 18,500 | 34.90 | 27,500 | 24,500 | 47.11 | 27,500 | 29,000 | 51.32 |
| Control | 117,500 | 3500 | 2.89 | 90,000 | 2000 | 2.17 | 80,000 | 5000 | 5.88 |

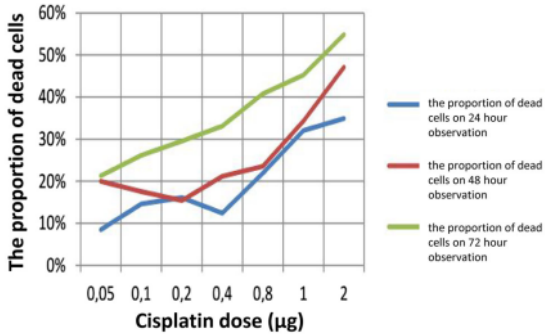


**Fig. 2** Graph of proportional correlation of KNF stem cell death at 48 h cisplatin exposure and different observation duration

cytotoxicity in NPC stem cells ($p < 0.05$). Data correlation of cisplatin exposure duration with death cell was obtained coefficient correlation − 0.357 and $p = 0.001$ indicating that there was the correlation between duration of cisplatin exposure with cytotoxicity profile on NPC stem cells which also significant ($p < 0.05$).

To determine an effective cytotoxic dose (EC50), a line drawn to indicate the dot of the cross point that results in 50% mortality from NPC stem cells from the graph in Fig. 3. The cutoff point from the line that correlates the dose and death cell by 50% on both charts were; both post-24-h exposures in Fig. 3a and post-exposure 48 h in Fig. 3b at approximately 1 µg dose, it is found that the effective cisplatin cytotoxic dose (EC50) against NPC stem cells was 1 µg.

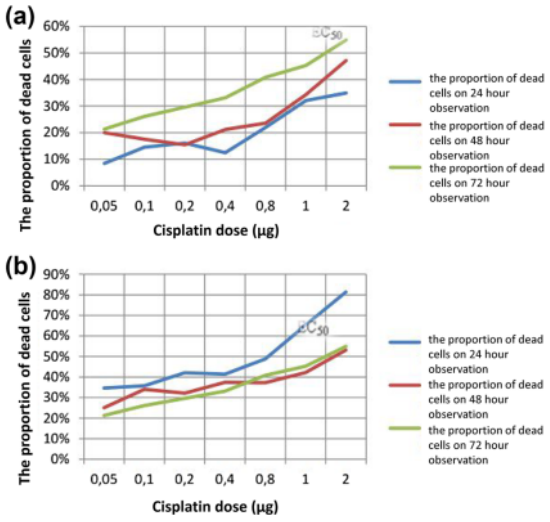


**Fig. 3** EC50 cisplatin dose graphs at 24 h (**a**) and 48 h (**b**)

## Discussion

In a population of tissues or cells, apoptosis and necrosis are two extremes of death cell [17]. Low cisplatin concentrations that correlated with apoptotic death cell and high doses cause the death cell due to necrosis [18]. Apoptosis is a response to cellular stress at the intensity of exposure below the necrotic threshold. High doses of cisplatin resulted in the damage of a number molecules that involved in the supply of cell energy adenosine triphosphate (ATP) and also the proteins directly involved or indirectly in the apoptotic process leading to the death of necrotic cells, as evidenced by the appearance of necrotic cell features at exposure to high doses of cisplatin cisplatin resistant keratinocyte tissue. Exposure to high doses of cisplatin causes the reduction of ATP cell levels resulting in severe ATP depletion. Then, it will cause a rapid metabolic collapse resulting in necrotic death cell. The fewer ATP depletions correlated with lower doses of cisplatin cause apoptosis by the release of mitochondrial cytochrome [14].

Some studies suggest two lag phase in cisplatin cell growth inhibition in accordance with the results of this study that within the first 6 h, no cisplatin inhibition effect was detected. It was estimated that in that period cisplatin accumulates and reaches the DNA genome to then express its pharmacological activity. After that period there was a rapid decrease in cell viability up to 20 post-exposure hours. The second lag phase of static cell/plateau growth occurs at 20–24 h, which estimated to occur due to inactivation of cisplatin by thiol compound, only by then, there will be a significant decrease in cell viability. At the length of exposure duration up to 48 h, there was an extensive membrane blockade of platelet function, the proportion of dead cells did not parallel with the drug content of assumption that saturation at the receptor has been achieved [18, 19].

In the 48-h duration of cisplatin exposure, cell proliferation has lasted for 2–4 generations of cells (assuming doubling the time of NPC cell line time in varies from 10.5 to 28.5 h) [20]. The highest level of cisplatin uptake (passive diffusion) at the early time, that the more death cell at the beginning of the duration exposure with the proliferation of cells have lasted 2 to 4 cells generations within 48 h, the number of dead cells becomes less than the new living cells resulting from the proliferation that occurs after the cell undergoes recovery [21].

The $EC_{50}$ indicator was the concentration or dose that required by a drug to achieve the desired effect of 50% in vitro. $EC_{50}$ for cytotoxicity means at concentrations of 50% cells showing the effect of death cell. Measurement of drug concentrations or doses usually follows a rapidly

increasing pattern of sigmoid curves in relatively small dose changes. The effective dose point mathematically determined by drawing the corresponding line that was more easily determined by a graph than a complex statistical equation [22].

A number of studies used both $EC_{50}$ and $IC_{50}$ indicators with similar results. Another study reported that the cisplatin dose of 0.5 μg in the cell line of NPC CNE1 cellular was damage but the cells still respond actively that characterized by the inhibition of cell growth in the early period of observation but then recovered. Shikanov et al., in 2011 conducted a study of cisplatin cytotoxicity on several cell culture types using $IC_{50}$ indicators obtained different values. In MBT cell culture of 0.8 μg/ml, in MBT-2 cells of 4.8 μg/ml and in Meth-AR-1 1.5 μg/ml cells [23].

Different doses that effect cytotoxic effects dissimilar between cell types because the mechanisms of apoptosis induced by cisplatin were unlike, and highly specific in each cell [18]. This difference might also be due to the doubling time difference between cell types, especially in the growth-regulated neoplastic cells. Other differences due to the live/dead cell measurement methods that used to determine cytotoxicity and cell proliferation. These were some of the factors that affect cytotoxicity but uninvestigated in this study.

## Conclusion

There was the correlation between the dose increased of cisplatin and cytotoxicity in NPC stem cells. Moreover, there was a correlation between the duration of cisplatin exposure and cytotoxicity in NPC stem cells. The effective dose of cisplatin resulting in a cytotoxic effect on NPC stem cells was by 1 μg at 24-h exposure duration.

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