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Preclinical report

Significance of scheduling on the cytotoxicity of radiation and cisplatin combination treatment in nasopharyngeal carcinoma cells

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The use of cisplatin as a potential radiosensitizer in nasopharyngeal carcinoma (NPC) has produced encouraging results in clinical trials. In order to provide information on improving the design of clinical treatments, we investigated the effect of cisplatin dose, and the time interval and sequence between administration of cisplatin and radiation on cell survival of two NPC cell lines, CNE1 and SUNE1. When cisplatin was applied first, an exposure time of 24 h resulted in up to 2.6-fold increase in cell death and 7-fold increase in radiation effect (cell survival after cisplatin/cell survival after cisplatin plus radiation) in the cisplatin–radiation combination treatment compared to the cells treated with cisplatin for 4 h. When radiation was applied first, a shorter interval time of 4 h followed by cisplatin treatment resulted in up to 3-fold increase in cell death and a 3-fold enhanced radiation effect over longer time intervals of 24 h. By changing the order of radiation and cisplatin treatment alone, a 2-fold difference in radiation effect was observed. The differential cytotoxicity was partially explained by the alterations in cell cycle distribution. Our results indicate the importance of scheduling the radiation and cisplatin combination regimens on the survival of NPC cells. [© 2002 Lippincott Williams & Wilkins.]

Key words: Cisplatin, nasopharyngeal carcinoma, radiation.

Introduction

Nasopharyngeal carcinoma (NPC) is a rare tumor in most parts of the world, but its incidence is high in Southern China. Although NPC is relatively sensitive to radiotherapy, recently evidence has shown that combination of radiation and chemotherapy achieved significantly better results, both in terms of tumor-free and overall survival.[1–4] In NPC, the most effective and widely used chemotherapeutic drug in combination with radiation is the DNA-damaging agent cisplatin.[3,5]

The clinical protocols associated with chemotherapy including cisplatin were highly variable in the sequence and time interval between irradiation and administration of drugs. So far, there is no report on the effect of scheduling in chemo-radiation combination therapy in NPC, either in clinical or experimental studies. It was shown in a clinical trial on breast cancer patients that the sequence of administration of chemo- and radiation therapy was important in influencing the outcome among patients at substantial risk of systemic metastasis.[6] *In vitro* studies also showed that cancer cell survival could be influenced by the time at which radiation was applied, the treatment sequence, and the doses of both radiation and chemotherapy.[7–11] Most of the experiments in which cisplatin was administered before radiation resulted in enhancement of radiation response both *in vivo* and *in vitro*.[8,12] However, in a recent study, when radiation was applied shortly before cisplatin, a significantly enhanced cell killing was observed in human ovarian carcinoma cells over other combined modalities.[10] In rat yolk sac-free cells, the synergistic effect of cisplatin and radiation was shown to be independent of the time of administration.[11] There was also evidence that radiation inhibited cisplatin-induced apoptosis when applied 24 h before cisplatin in glioma cells.[13] Evidence to date indicates that the synergistic response of cisplatin and radiation combination may depend on the combination regimens and the type of tumors.

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The objective of the present *in vitro* study was to investigate the effect of sequence and exposure time on the cytotoxicity in cisplatin–radiation combination treatment on two NPC cell lines, CNE1 and SUNE1. In addition, the cell cycle distribution was studied in the cells treated with various tested protocols to evaluate the possible role of cell cycle alterations on the outcome of the combined treatment. Our results confirmed the importance of timing and sequencing in determining the cytotoxicity of radiation–cisplatin combination treatment in NPC cells.

Materials and methods

Cell culture conditions and drug treatment

Human NPC cell lines CNE1 and SUNE1, derived from poorly differentiated NPC in Chinese patients,[14] were maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 2 mM L-glutamine and 10% (v/v) fetal calf serum. All cultures were maintained *in vitro* for less than 10 passages continuously. Cisplatin was purchased from David Bull Laboratories (Victoria, Australia). The cells were irradiated using a Gamma Cell 1000 Elite machine at a dose rate of 14 Gy/min. The energy source was ^{137}Cs.

Colony forming assay

Exponentially growing cells were detached from the cell culture surface by incubating with trypsin–EDTA [0.25%. (w/v)/2%(w/v)] for 3 min at 37°C and a single-cell suspension was produced. Viable cell number was assessed using a hemocytometer by counting the number of cells excluding Trypan blue [0.1% (w/v) in PBS]. An appropriate dilution of the cells was made to produce the final cell number required for each colony forming experiment in six-well plates; 1000 cells were plated to result in 150–200 colonies per well in control wells after 8–10 days culture. Five doses of either radiation or cisplatin were used in each survival curve. Colonies that consisted of more than 50 cells were scored and compared to the untreated controls. Two wells were used for each concentration and each experiment was repeated at least 3 times. The cell survival curves were drawn by plotting the means from at least three experiments and the SD as error bars.

Cisplatin and radiation combination treatment

Cells (5×10^5) were plated in three 6-cm dishes 24 h before cisplatin treatment (Dishes A–C). Dish A was counted and 1000 cells were plated in two wells of a six-well plate as untreated control. The remaining cells in dish A were irradiated with 1 Gy radiation and 1000 cells were plated in each well in six wells (two of them were used as radiation alone). Cisplatin was then added 4 (RT4CP) and 24 (RT24CP) h later in two wells, respectively. Dish B was treated with cisplatin for 4 h and plated in two wells (CP4hr), the remaining cells were irradiated at 1 Gy and plated as in the controls (CP4RT). Dish C was exposed to

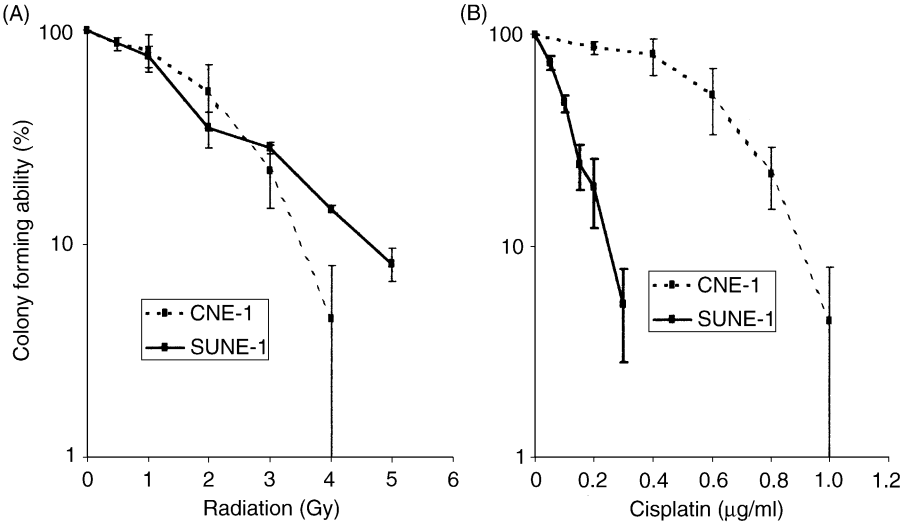

Figure 1. Dose–response curves of CNE1 and SUNE1 cells to radiation and cisplatin. Colony forming assays were performed as described in Materials and methods. Each data point represents the mean from at least three experiments and the error bars indicate the SD.

cisplatin for 24 h and plated (CP24hr), the remaining cells were irradiated at 1 Gy and plated (CP24RT). After 8–10 days, the cells were fixed in 70% ethanol, stained with 10% Giemsa stain and the colonies were counted as described in 'Colony forming assay'.

Analysis of cytotoxicity

Two-sided Student's *t*-test was carried out to compare the cell survival between different treatment modalities. The difference was considered significant when $P < 0.05$. The radiation effect (RE) was defined as the ratio between cell survival after cisplatin and cell survival after cisplatin and irradiation treatment.[8]

Cell cycle analysis

Cells (5×10^5) were treated with different combinations of cisplatin and radiation, and harvested at 24 and 48 h post-treatment. The cells were trypsinized, fixed in cold 70% ethanol for 24 h and stored at 4°C. Before testing, the fixed cells were washed with PBS and treated with RNase (0.1 mg/ml), and stained with propidium iodide (50 μg/ml) for 30 min at 37°C. Cell cycle analysis was performed on an Epics analyzer using ModFit LT2.0 software (Coulter, Hialeah, FL).

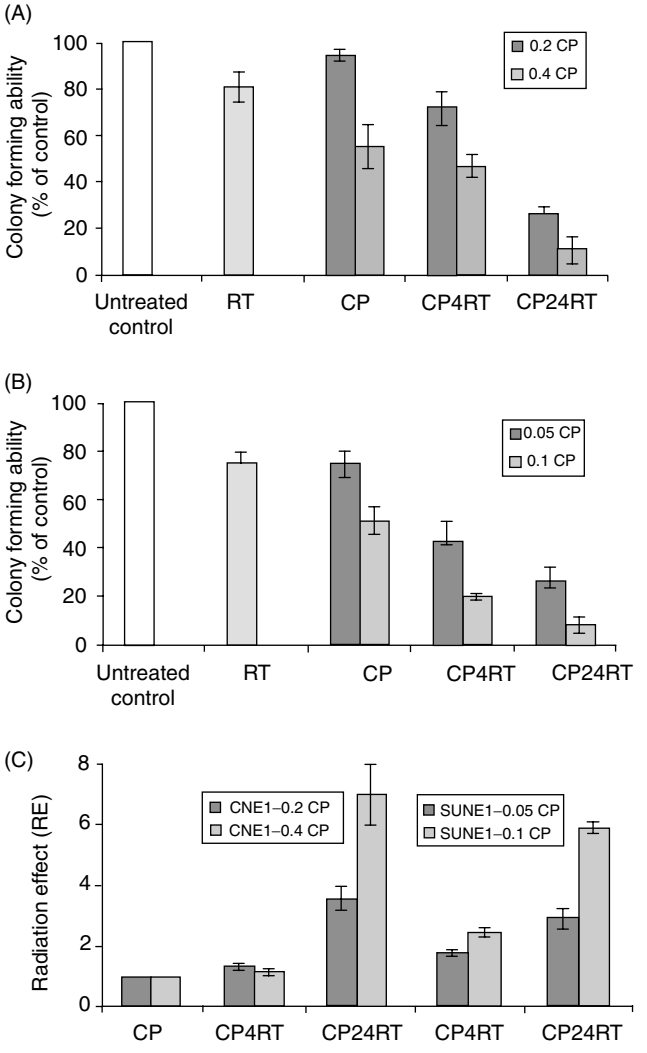

Figure 2. Colony forming ability of CNE1 (A) and SUNE1 (B) cells after exposure to 1 Gy radiation (RT), cisplatin (CP), cisplatin for 4 h then radiation (CP4RT) and cisplatin for 24 h then radiation (CP24RT). Colony forming assays were performed as described in Materials and methods. Each data point represents the mean from at least three experiments and the error bars indicate the SD. (C) RE values derived from different combination regimens. The RE was defined as the ratio between cell survival after cisplatin and cell survival after cisplatin and irradiation.[8]

Each data point represented the mean of at least two experiments and the error bars represented the SD.

Results

Determination of radiation and cisplatin doses to be used in combination studies

The effect of radiation and continuous cisplatin exposure on two NPC cell lines, CNE1 and SUNE1, was studied using the colony forming assay. These two cell lines showed similar sensitivity to radiation (Figure 1A), but CNE1 is 4-fold more resistant to cisplatin than SUNE1 cells (Figure 1B), comparing their IC_{50} doses (a dose that inhibits 50% colony forming ability as compared to the untreated controls). A radiation dose of 1 Gy was used in the combination studies, which resulted in approximately 20% inhibitory effect on colony forming ability in both cell lines. Due to their differential sensitivity to cisplatin, different doses of cisplatin were used for CNE1 (0.2 and 0.4 μg/ml) and SUNE1 (0.05 and 0.1 μg/ml) to result in similar cytotoxicity of approximately 80 and 50% colony forming reduction on both cell lines.

Effect of cisplatin exposure time on radiation response

CNE1 and SUNE1 cells were exposed to two concentrations of cisplatin, respectively, first, and then given 1 Gy of radiation at 4 (CP4RT) and 24 (CP24RT) h post-exposure. A significant difference in colony forming ability was observed in the cells treated with the two schedules (Figure 2A and B, $p < 0.05$). An up to 7-fold enhanced RE was found in the cells treated with cisplatin for 24 h before radiation (CP24RT) over CP4RT combination (Figure 2C). The changes in RE values were not as

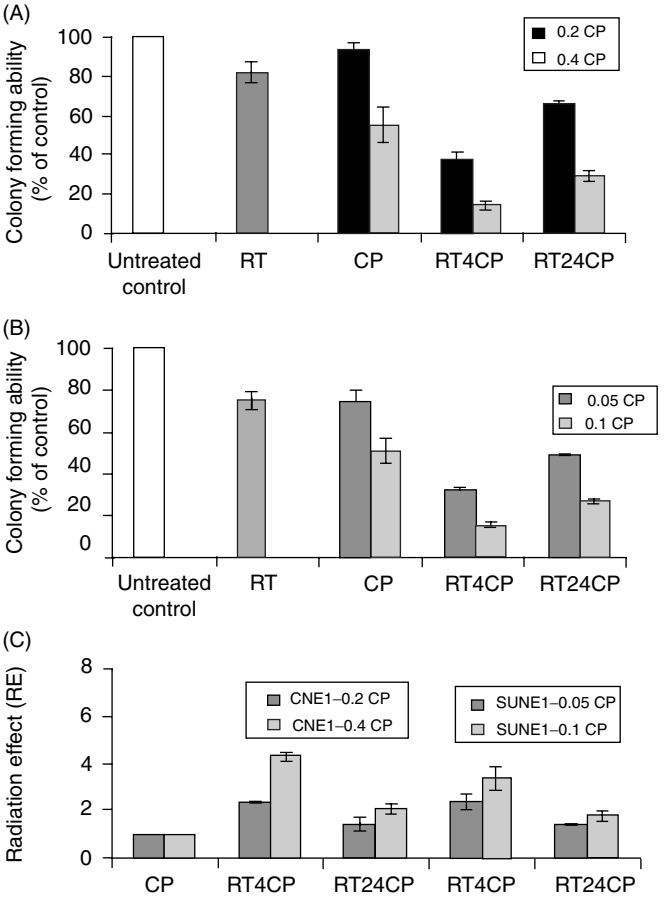

Figure 3. Colony forming ability of CNE1 (A) and SUNE1 (B) cells after exposure to 1 Gy radiation (RT), cisplatin (CP), radiation first then cisplatin 4 h later (RT4CP) and radiation first then cisplatin 24 h later (RT24CP). Colony forming assays were performed as described in Materials and methods. Each data point represents the mean from at least three experiments and the error bars indicate the SD. (C) RE values derived from different combination regimens.

significant in SUNE1 cells as in CNE1 cells; however, a similar correlation was observed. The effect of cisplatin on radiation enhancement was dependent on exposure time on both cell lines.

Effect of radiation interval time followed by cisplatin on radiation response

The two NPC cell lines were irradiated at a dose of 1 Gy, and two concentrations of cisplatin were added 4 (RT4CP) and 24 (RT24CP) h after radiation. When cisplatin was added at 4 h post-radiation, a greater increase in cell death was observed compared to the combination in which cisplatin was given at 24 h after radiation in both cell lines (Figure 3A and B, $p < 0.05$). The RE values were significantly higher in the cells treated with cisplatin 4 h after radiation than the cells treated with cisplatin 24 h after radiation (RT4CP versus RT24CP) (Figure 3C, $p < 0.05$).

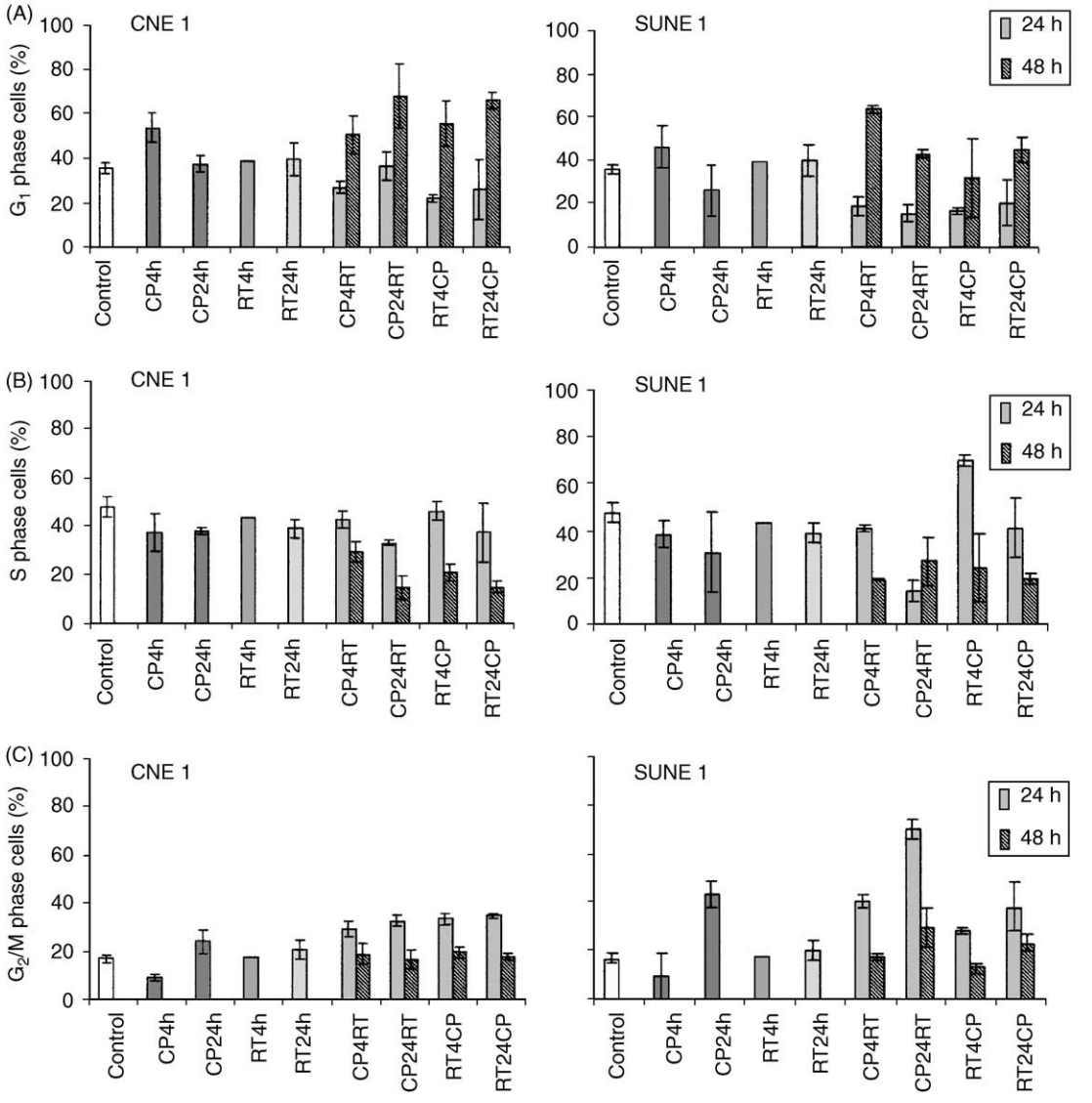

Figure 4. Cell cycle alterations following radiation, exposure to cisplatin and their combined treatments in CNE1 and SUNE1 cells. Percentage of G_1 (A), S (B) and G_2/M (C) phase cells were plotted against cells treated with different combinations. The cells were treated with cisplatin (0.4 μg/ml for CNE1 cells and 0.1 μg/ml for SUNE1 cells) at 4 (CP4h) and 24 (CP24h) h post-exposure. The irradiated cells were also analyzed at the post-treatment times of 4 (RT4h) and 24 (RT24h) h. The cells treated with the combination of cisplatin and radiation (CPRT or RTCP) were collected at 24 and 48 h post-treatment. Each data point represents the mean of at least two experiments and the error bars indicate the SD.

Effect of combination sequence on radiation response

Significant differences in colony forming ability were observed by changing the order of radiation and cisplatin treatment given at 4-h intervals (CP4RT versus RT4CP). Radiation applied first followed by cisplatin at 4 h post-irradiation resulted in a significant decrease in colony forming ability compared to the same combination when cisplatin was added first (Figures 2 and 3, $p < 0.01$). The RE values were also significantly increased in the cells treated with RT4CP schedule than the CP4RT schedule (up to 2.5-fold, Figures 2C and 3C, $p < 0.05$). At the 24-h interval, the sequence of administration of cisplatin and radiation did not show any significant effect on the colony forming ability in these cells.

Alteration of cell cycle distribution following different treatments

To study the possible role of cell cycle alteration on the outcome of the combined treatments, flow cytometric analysis was performed following irradiation, exposure to cisplatin and their combinations at 4, 24 or 48 h post-treatment on both cell lines (Figure 4).

Cisplatin alone induced an increase in the percentage of G_1 phase cells at 4 h post-exposure (Figure 4A, CP4h). At 24 h post-exposure, the cisplatin-treated cells were arrested in the G_2/M phase of the cell cycle (Figure 4C, CP24h). However, significant cell cycle changes were not observed in cells treated with radiation alone compared to the untreated controls (Figure 4, RT4h and RT24h).

All combined treatments induced a decreased percentage of G_1 phase cells at 24 h post-treatment compared to the untreated controls and the percentage increased by 80–150% at 48 h compared to the 24 h time point (Figure 4A). The percentage of S phase cells decreased in all but one combination (CP24RT, SUNE1) at 48 h post-treatment compared to the 24 h time point (Figure 4B). Alterations in the percentage of G_2/M phase cells were observed in most of the combined treatments and an approximately 300% increase was found in CP24RT (SUNE1) at 24 h post-exposure when compared to the untreated controls. At 48 h after treatment, the percentage of G_2/M phase cells decreased, indicating that the cells had overcome the cell cycle arrest.

Discussion

Although NPC is considered to be one of the most radiosensitive head and neck cancers, the combination of radiation and chemotherapy has been shown to result in a much higher response rate in patients with advanced and metastatic disease over single agents alone.[2] Despite numerous combination chemotherapy trials performed on NPC, clear conclusions cannot be drawn in the literature on the effect of different regimens on survival. Therefore the main objective of this study was to compare different multimodality treatments to study the cytotoxic response on two NPC cell lines. Our results showed that when radiation was combined with a single chemotherapeutic drug, treatment scheduling was important in determining cytotoxicity.

It must be emphasized that the present experiments were not specifically designed to study the mechanisms of drug–radiation interaction. Our results showed that the time and sequence of cisplatin treatment had a significant effect on radiation response. When cisplatin was applied first, the RE was dependent on exposure time as the exposure time of 24 h (CP24RT) before radiation resulted in up to 2.6-fold decrease in colony forming ability and 7-fold increase in RE values than in the cells treated with cisplatin for 4 h (CP4RT) (Figure 2). However, when radiation was applied first, a shorter interval time followed by cisplatin (RT4CP) yielded a significantly lower colony forming ability and higher RE values than longer intervals (RT24CP) (Figure 3). By changing the order of the radiation and cisplatin treatment alone (CP4RT to RT4CP), increased RE values were also observed (Figures 2C and 3C). Our data clearly demonstrated the importance in scheduling the radiation and cisplatin combination regimens. With regard to the clinical situation, our data suggests that if radiation is applied after cisplatin, it should not be given immediately after cisplatin and an interval time of 24 h would lead to higher toxicity. However, if radiation is applied first, cisplatin should be administered soon after (such as 4 h later).

Our results are partially in agreement with recent experimental studies showing that maximal toxicity was observed in cell lines treated with radiation shortly before cisplatin exposure (RTCP).[8,10,15] One recent study showed that radiation conferred resistant to cisplatin when applied 24 h before cisplatin (RT24CP) on glioma cells,[13] which partially correlated with the present evidence that the RT24CP schedule resulted in a significantly higher colony forming ability than the RT4CP schedule (Figure 3). In the previous studies, when cisplatin was

administered before radiation (CPRT), however, there were no consistent results on the effect of time and sequence of combination therapy on radiation response in other types of human cancer.[7,9,10,15,16] However, we found a significant difference in cytotoxicity between CP4RT and CP24RT schedules (Figure 2). It is possible that the cellular response to cisplatin and radiation combination therapy is cell-type specific.

The alterations in cell cycle distribution may explain the differential cytotoxicity between CP4RT and CP24RT treatments. At 24-h post exposure time (CP24h), cisplatin induced a cell cycle G_2/M phase block compared to CP4h (200–400% increase in G_2/M phase cells) (Figure 5C), the phase in which cells have been reported to be the most sensitive to radiation.[17] However, after 4 h of exposure in cisplatin, the percentage of G_1 phase cells was increased compared to CP24h (50–70% increase) (Figure 4A), the phase in which cells have been shown to be relatively resistant to radiation.[17] Therefore, when radiation was applied, the population containing a relatively high percentage of G_2/M phase cells (CP24h) was more sensitive than the population containing a high percentage of G_1 phase cells (CP4h). At the moment we cannot correlate the cell cycle alterations to cell survival between RT4CP and RT24CP combinations as there were no significant changes in cell cycle distribution at 4 and 24 h post-radiation when cisplatin was applied (Figure 4). In previous studies, the supra-additive effect of similar modality could not be explained by the alterations in cell cycle distribution in human ovarian carcinoma cell lines.[10] Currently, we are investigating the role of DNA damage-related proteins such as p53, p21, MDM2, Bcl-2 and Bax in the differential cytotoxicity of the two NPC cell lines.

Conclusion

In this *in vitro* study, we showed that there was a difference in radiation effect when the same doses of cisplatin and radiation were applied with different combination regimens in nasopharyngeal carcinoma cells. In addition, the alterations on cell cycle distribution may play a role in the differential cytotoxicity between certain combination treatments. Our results provide the first data on the effect of scheduling in chemo-radiation combination treatment in cytotoxicity on nasopharyngeal carcinoma cells. It is hoped that the present data may lead to a possible clinical trial aimed at improving the efficiency of chemo-radiation combination therapy in nasopharyngeal carcinoma cells.

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