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Assessing the relative biological effectiveness of high-dose rate ^{60}Co brachytherapy alone and in combination with cisplatin treatment on a cervical cancer cell line (HeLa)

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ABSTRACT

Objective: Cervical cancer is the fourth common malignancy in women for which combinational therapy is the main treatment regimen. This study aimed to determine the effects of ^{60}Co γ-ray high dose rate brachytherapy on the survival of HeLa cells with respect to X-ray of 6 MV linear accelerator (EBRT, external beam radiation therapy) as a comprehensive radiobiological concept. Furthermore, alterations in relative biological effectiveness (RBE) values were examined in combined treatments of cisplatin and radiation.

Methods: Cell survival was evaluated following different treatments by clonogenic assay. The synergistic effects of cisplatin and X-ray were evaluated in terms of the combination index (CI). Moreover, fold change reduction of each treatment in combination therapy was determined by evaluating the dose reduction index (DRI). RBE values were also determined after different treatments.

Results: Maximum values of DRI for cisplatin and radiation were 128 and 2.2-fold reduction when used in combination, respectively. As a result of the CI and DRI values, pretreatment with 2 μg/ml cisplatin for 2 h was selected as the most effective concentration and time, and it was used in RBE evaluation. The RBE values at 50% cell survival after treatment with radiation alone and also combined with cisplatin were measured as 1.308 and 1.553, respectively. These RBE values indicate a more destructive effect of γ-radiation brachytherapy than X-ray EBRT.

Conclusion: The results of combined treatment indicate a remarkable synergism. Furthermore, our data determined that ^{60}Co γ-ray is more effective than EBRT X-ray to induce cell death in HeLa cells.

1. Introduction

Cervical cancer ranks as the fourth most frequently diagnosed malignancy and the fourth leading cause of cancer related deaths in women. Most cases of this disease occur in less developed countries (Bray et al., 2018). Current treatments for cervical cancer include radiotherapy and platinum-based chemotherapy. One of the treatment limitations is acquired radio-resistance as well as overall dose limitation, which means that the dose level used for treating a tumor must have minimal damage to healthy tissues (Su et al., 2012). There are two types of radiotherapy, external beam radiotherapy (EBRT) and brachytherapy.

Depending on the stage of the disease, patients may receive EBRT in which a large volume of tissues are irradiated, so the effective dose that affects the target tissue decreases and damage to adjacent tissues would increase. Because of this limitation in dose escalation, to deliver the required total dose and achieve local control, brachytherapy could be considered as a better option (Mahantshetty and Krishnatry, 2013; Moding et al., 2013; Greenhalgh et al., 2014; Sharma and McNeill, 2009). Since the effect of radiation is directly related to the distance from the source, by reducing the distance in the intracavitary method, brachytherapy is considered as an accurate and conformal therapy (Hoskin and Bownes, 2006; Podgorsak, 2005). Cobalt-60 (^{60}Co) is

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applied in high-dose rate (HDR) brachytherapy as a radiation source, which is highly common in developing countries, for its long half-life and cost-effectiveness (Podgorsak, 2005; Ntekim et al., 2010).

Although radiotherapy is widely used in cancer treatment, 20–40% of patients treated with conventional radiation therapy undergo the disease recurrence, and unfortunately they have a very poor prognosis (Beadle et al., 2010). Possible reasons to justify this ineffectiveness include inadequate delivery dose to all parts of the target tumor and also reduced dose of the radiation (Beadle et al., 2010; Kidd et al., 2010). In the application of radiotherapy, it is crucial to evaluate the biological effects of the radiation used and effective doses for treatment. The relative biological effectiveness (RBE) is the most important characteristic of radiation in determining the treatment outcome. RBE is the dose ratio of two different radiation sources that yield the same biological impact, such as cell survival fraction which is examined using clonogenic assay (Nikjoo and Lindborg, 2010; Valentin et al., 2003). As a result, the assessment of RBE is crucial for each type of radiation and different cancer cell lines.

Ionizing radiation has two types of biological effects including lethal and sublethal lesions, which consist of non-repairable and repairable damages, respectively (Mazeron et al., 2002). In radiobiological studies, cell death after radiation is described by the linear-quadratic (LQ) model: survival fraction $= \exp(-(\alpha D + \beta D2))$ (Ang et al., 1984). α is the linear parameter and represents lethal damage, while β is the quadratic parameter indicating sublethal damage. Furthermore, the influence of chemotherapy agents on radiation dose survival curves can adequately be analyzed with the use of the LQ model (Barendsen, 1990, 1997; Franken et al., 2011).

Studies have shown that the combination of radiation and a platinum-based chemotherapy drug, such as cisplatin, (cis-diamminedichloro platinum (II), CDDP) would be more effective in the treatment of advanced and metastatic cervical cancers, and improves the treatment efficacy (Greenhalgh et al., 2014; Serkies and Jassem, 2005). A study on HeLa cells, a cervical carcinoma cell line, indicated that synergism can be observed between chemotherapy and X-ray radiotherapy. In this manner, cisplatin acts as a radiosensitizer and makes the cancer cells sensitive to radiation that would otherwise be resistant (Monk et al., 2002). The synergism between radiation and chemotherapy can be represented by combination index (CI) (Chou et al., 1983) and dose reduction index (DRI) parameters. These kinds of treatment modalities are currently carried out in the clinic. Although the results of combination therapy are better than radiation alone, the combination regimen has greater side effects and overall survival is not satisfactory (Maduro et al., 2003). To deal with this problem, *in vitro* experiments would help to predict the treatment outcome. Among all *in vitro* analyses, clonogenic assay demonstrates a better correlation between *in vitro* sensitivity to radiation and recurrence in patients (West et al., 1993). This study aimed to determine the effects of ^{60}Co γ-ray HDR brachytherapy on HeLa cells colony formation in comparison with X-ray of 6 MV linear accelerator (EBRT) in terms of RBE and α/β ratio. Furthermore, the synergism of combined treatment was evaluated, and RBE and α/β ratio alterations were examined in combination with cisplatin pre-treatment followed by radiation.

2. Materials and methods

2.1. Cell culture and viability assessment

HeLa cells, a human cervical carcinoma cell line, were purchased from Pasteur Institute, Tehran, Iran and cultured in Roswell Park Memorial Institute (RPMI 1640) medium (Life Technologies, UK) supplemented with 10% (v/v) fetal bovine serum (FBS) (Life Technologies, UK) at 37 °C with 5% CO_2 in humidified air. Cells were passaged with 0.25% trypsin-1 mM EDTA (ethylenediaminetetraacetic acid) when required (Life Technologies, UK). To ascertain the optimal cisplatin pre-treatment concentration, at first, the IC_{50} values of cisplatin on HeLa cells were determined by MTT assay. For this purpose, HeLa cells were detached using trypsin and seeded in 96-well plates (7.0×10^4 cells/ml). After 24 h incubation when confluency reached 70–80%, cells were treated with 25, 12.5, 6.25, 3.125, and 1.5 μg/ml cisplatin for 24, 48, and 72 h, as required. After each time interval, MTT (5 mg/ml) (Sigma Aldrich, Germany) was added to each well at the final ratio of 10% (v/v) for 4 h. Then, the media were replaced with 200 μl DMSO (dimethyl sulfoxide, Merck, Germany), and absorptions were read at 545 nm wavelength with an ELISA reader (Awareness Technology Incorporation, USA).

2.2. Clonogenic cell survival assay

The colony-forming assay for irradiation experiments was carried out as described previously (Franken et al., 2006). This assay can be performed in two different ways, plating before or after irradiation. In this study, cells were plated after cisplatin and radiation treatments. To do so, cells were grown in 25 cm^2 flasks to reach 90% confluency, then for cisplatin and irradiation experiments, they were cultured in 96-well plates at the concentration of 7.0×10^4 cells/ml. After they reached 80–90% confluency, for cisplatin alone or pre-treatment before irradiation, cells were treated with different concentrations of cisplatin (1 and 2 μg/ml) for various time intervals (1, 2, 3 and 4 h), as required. Afterwards, the media were replaced with a drug-free medium and cells were left for 24 h in cisplatin alone groups, or irradiated. The day after treatments (cisplatin alone or with irradiations), cells were detached using trypsin and a specific number of cells (depending on the treatments; 100–10000 cells/well) were seeded in 6-well plates for colony formation and the plates were left at 37 °C with 5% CO_2 in a humidified incubator for 10–14 days.

2.3. Fixation and staining of colonies

After 10–14 days, colonies were rinsed with 10% phosphate-buffered saline (PBS) (Sigma, Germany), fixed in 70% methanol (Merck, Germany) for 5–7 min followed by staining with Giemsa (Merck, Germany) for 30 min. Plates were then carefully rinsed with water and left to dry at room temperature. Colonies with more than 50 cells were counted and used for calculation of survival fraction (Niyazi et al., 2007).

2.4. Survival fraction

Obtained data from colony counting were applied for calculation of plating efficiency (PE) and survival fraction (SF) based on the following formulas (Franken et al., 2006):

$$PE\% = \frac{number\ of\ colonies\ formed}{number\ of\ seeded\ cells} \times 100$$

$$SF = \frac{number\ of\ colonies\ formed\ after\ treatment}{number\ of\ seeded\ cells\ \times\ PE}$$

Obtained SF values were analyzed with GraphPad Prism 6.07 software based on the LQ model to achieve survival curves, α, and β parameters.

3. Irradiation

3.1. External beam radiation

X-ray irradiation was done using a 6 MV photon beam from Siemens ARTIST linear accelerator using a radiation field of 10×15 cm^2 covering the whole plate with enough margin. The cells were irradiated with 2 Gy–8 Gy doses with the beam calibrated to deliver 100 cGy to the maximum depth with 100 MU according to the TRS 398-IAEA guidelines (Almond et al., 1999).

3.2. Brachytherapy

For brachytherapy, irradiation of cells was performed using a ^{60}Co high dose rate brachytherapy afterloader unit from Bebig Ecker & Ziegler (Berlin-Germany) clinically used at Reza Radiotherapy and Oncology Center in Mashhad. An intraluminal flexible nasopharynx catheter (D = 1.65 mm, L = 300 mm) was used for irradiation.

Determination of the irradiation time and dosimetry were calculated using the HDR Plus treatment planning system based on the Task Group 43-(AAPM-TG43) formalism (Rivard et al., 2004). The catheter was set under the plate so that the first well position set on the desired well and the source was sent to the planned position to deliver the doses of 2, 4, 6, and 8 Gy to the cells.

3.3. Relative biological effectiveness

In this study, EBRT was the reference radiation (D_r) and [60]Co γ-ray was considered as a test (D_t). The RBE ratio was calculated as RBE = D_r/D_t at 50% cell survival (Nikjoo and Lindborg, 2010; Valentin et al., 2003).

3.4. Synergistic effects

CompuSyn 1.0 software (Chou, 2006) was applied to calculate the synergistic effects of 1 and 2 μg/ml cisplatin pre-treatments for 1 and 2 h with various doses of 6 MV linear accelerator X-ray *in vitro*. CI and DRI were reported as synergistic parameters.

4. Results

4.1. MTT assay

After treatment of HeLa cells with different concentrations of cisplatin, the ELISA reader output (optical density) was analyzed with GraphPad Prism 6.07 software to achieve dose-response curves and IC_{50} values, which were calculated as 3.78, 2.32 and 1.90 μg/ml (12.58, 7.72, 6.32 μM) after 24, 48 and 72 h treatments, respectively (Fig. 1).

4.2. Clonogenic assay

To evaluate the colony formation efficacy after treatment with cisplatin, HeLa cells were treated with 3 concentrations close to cisplatin IC_{50} values (2, 4, and 6 μg/ml) and cell survival was calculated after 1, 2, 3 and 4 h of treatments. Survival data showed that treatment with 4 and 6 μg/ml cisplatin as well as 3 and 4 h interval times greatly reduced viability of HeLa cells (Fig. 2), so these concentrations and interval times were omitted from the following experiments and the efficacy of treatment with 1 and 2 μg/ml cisplatin after 1 and 2 h of treatments were
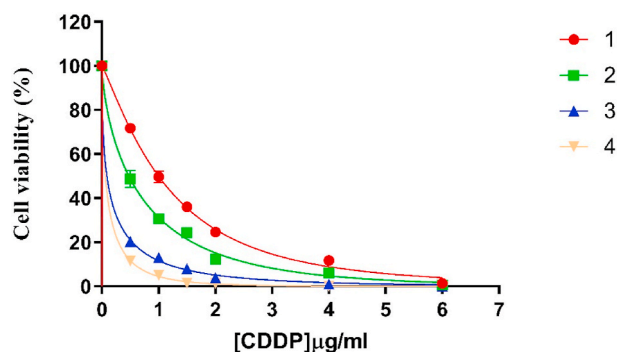
Fig. 2. Dose-response curves of HeLa cells obtained from clonogenic assay after treatment with different concentrations of CDDP (cisplatin) at 1, 2, 3, and 4 h interval times. The results are indicated as mean from 3 repeats with SEM.

further analyzed. These concentrations and interval times have also been used in other literature (Yang et al., 2009) similar to our experiments. To further analyze the effects of X-ray radiation on HeLa cells pre-treated with cisplatin, the combination effects were verified by clonogenic assay. Our results indicated that the plating efficiency of HeLa cells was between 70 and 80% in all experiments. The clonogenic survival of HeLa cells is depicted in Fig. 3 after irradiation with X-ray. The curves are plotted as survival fraction to radiation doses for the two interval times. The results indicated that 2 h cisplatin pre-treatment has relatively higher destructive effects on HeLa cells.

5. Synergistic effects of EBRT and cisplatin combined treatments

5.1. Combination index

In this research, the CI parameter was calculated according to the Chou method using CompuSyn software to evaluate the synergism of cisplatin and X-ray in combined treatments. CI values calculated for combinational treatments of X-ray radiation with 1 and 2 h cisplatin pre-treatments are shown in Table 1. The values of CI < 1.0, CI = 1.0 or CI > 1.0 indicate the synergistic, additive and antagonistic effects of the combined treatments, respectively (Rivard et al., 2004; Yang et al., 2009). According to the results, using 2 μg/ml cisplatin with different doses of EBRT represents more synergism. Furthermore, the 2 h time interval of cisplatin pre-treatment shows lower values of CI which indicate higher synergism at this time point.

5.2. Dose reduction index

DRI parameter infers the amount of dose reduction in combination therapy as compared with a single treatment. DRI values are shown in Table 2 (1 h cisplatin pre-treatment) and Table 3 (2 h cisplatin pre-treatment). The DRI values indicate the beneficial effects of combined treatments. Higher DRI values (DRI > 1) represent a greater reduction in treatment doses for the desired therapeutic effects (Chou, 2006). The highest DRI for cisplatin was obtained at the concentration of 2 μg/ml for 2 h and 8 Gy, which showed a 128-fold reduction. Furthermore, the highest DRI of X-ray was observed at 2 μg/ml cisplatin concentration for 1 h and 2 Gy, which was 2.2 times dose reduction.

5.3. Brachytherapy

In Veigel et al. study, at low doses, the cellular response is not significantly different from the homogeneous pattern. At higher doses, however, the subpopulation of the cells that receives less than the average dose, modifies the shape of the survival curve and creates a lower slope (Veigel et al., 2017). In this study, the data of 2 Gy dose were consistent with the study by Veigel et al. and the obtained results
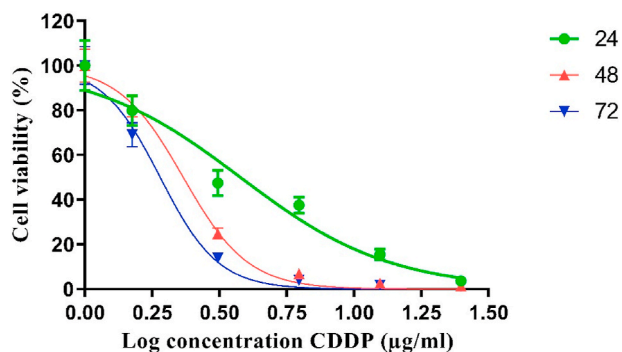

Fig. 1. Dose-response curves of HeLa cells as obtained by MTT assay after treatment with different concentrations of CDDP (cisplatin) at 24, 48, and 72 h. The results are derived as mean from 3 replicates with SEM.
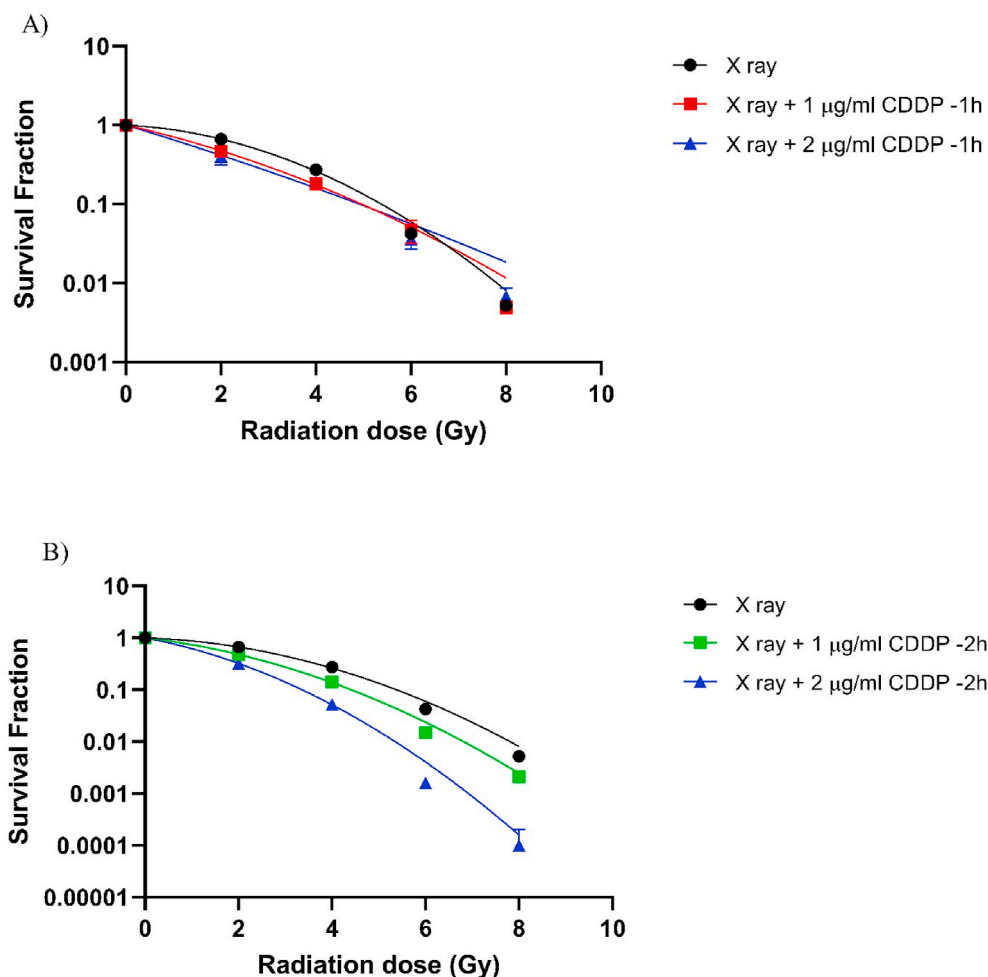
Fig. 3. Clonogenic cell survival curves of HeLa cells after 1 (A) and 2 (B) h pre-treatments with 1 and 2 μg/ml CDDP (cisplatin) and different X-ray doses compared to single exposure (black) curves. Data points represent mean ± SEM for three independent experiments.

Table 1
CI values for combined treatment of HeLa cells with 1 and 2 h CDDP (cisplatin) pre-treatment and X-ray radiation. *Fraction of affected cells.

[CDDP] (μg/ml)	Radiation dose (Gy)	Combination with 1 h cisplatin pre-treatment		Combination with 2 h cisplatin pre-treatment	
		Fa*	CI	Fa*	CI
1.0	2.0	0.779	1.00516	0.854	0.79514
1.0	4.0	0.91	1.08481	0.956	0.90429
1.0	6.0	0.976	1.01226	0.985	0.93648
1.0	8.0	0.998	0.68825	0.997	0.78906
2.0	2.0	0.9	0.91756	0.963	0.68797
2.0	4.0	0.953	1.00405	0.994	0.62939
2.0	6.0	0.991	0.81454	0.998	0.63230
2.0	8.0	0.998	0.70375	0.999	0.66301

Table 2
DRI values for combined treatment of HeLa cells with 1 h CDDP (cisplatin) pre-treatment and X-ray radiation. * The Fa values in this table are obtained from those in Table 1.

Fa*	[CDDP] (μg/ml)	Radiation dose (Gy)	DRI CDDP	DRI Radiation
0.779	2.24541	3.57269	2.24541	1.78634
0.91	4.58820	4.61434	4.58820	1.15358
0.976	11.7904	6.46937	11.7904	1.07823
0.998	64.5524	11.8913	64.5524	1.48641
0.9	4.23993	4.48575	2.11997	2.24287
0.953	7.35477	5.46361	3.67738	1.36590
0.991	23.1675	8.23932	11.5838	1.37322
0.998	64.5524	11.8913	32.2762	1.48641

showed a decrease in cell survival in radiation alone and in combined treatment with cisplatin (Fig. 4). Due to the sharp dose fall-off in brachytherapy, the target is usually covered by a heterogeneous dose distribution affecting the shape of the cell survival curve. However, at doses higher than 2 Gy, due to the steep gradient of doses in this method, the cell survival curve did not represent a logical pattern. Therefore, further investigations are needed to find an appropriate arrangement for homogeneous brachytherapy irradiation *in vitro*.

5.4. RBE

To calculate RBE, the doses of EBRT (reference dose) and brachytherapy that resulted in 50% viability were selected from the curves presented in Figs. 3B and 4. The RBE values were then calculated for single treatment with radiation (RBE $_r$) as 1.308, and for combined treatment, it was about 1.553. These data show that the combinational therapy affects RBE value.

$$RBE_r = {}^{2.617}/2 = 1.308$$

$$RBE_{r+CDDP} = {}^{1.864}/1.2 = 1.553$$

Table 3
DRI values for combined treatment of HeLa cells with 2 h CDDP (cisplatin) pre-treatment and X-ray radiation. * The Fa values in this table are obtained from those in Table 1.

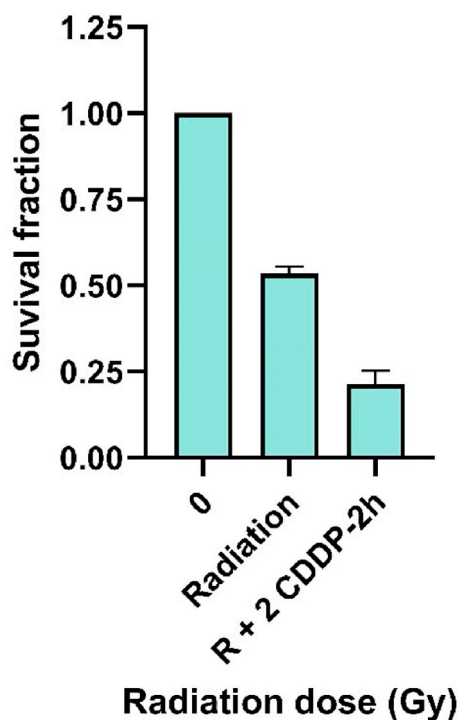| Fa*	[CDDP] (μg/ml)	Radiation dose (Gy)	DRI CDDP	DRI Radiation
0.854	1.81367	4.4612	10.2774	1.43300
0.956	4.69565	5.59124	15.9652	1.18814
0.985	10.4691	7.34350	25.4250	1.11464
0.997	33.9175	10.9517	64.0669	1.29291
0.963	5.35235	5.84571	22.7475	1.55277
0.994	20.4763	9.22489	58.0163	1.63357
0.998	45.5404	12.1058	96.773	1.60780
0.999	75.3249	14.3648	128.052	1.52626

Fig. 4. Clonogenic cell survival assay for HeLa cells after 2 h pre-treatment with 2 μg/ml of CDDP (cisplatin) and 2 Gy dose γ-ray of brachytherapy compared to single exposure. Data points represent mean ± SD for three independent experiments.

5.5. Therapeutic rate (α/β ratio)

Survival data for HeLa cells were extracted using the LQ survival model. According to the LQ model, the α/β ratios were estimated after EBRT treatment plans. At first, for the EBRT treatment the α/β ratio was 1.057 Gy, and for the combined treatment with 1 and 2 μg/ml cisplatin after 2 h were calculated as 3.785 and 4.455 Gy, respectively.

6. Discussion

Recent studies have shown that the use of radiation therapy in combination with chemotherapy using platinum-based drugs such as cisplatin can increase survival and improve the effectiveness of treatments in the advanced and metastatic stages of cervical cancer (Brezar et al., 2020; Furqan et al., 2019; Cihoric et al., 2017; Helfenstein et al., 2019). Although both of these therapeutic approaches are known as effective treatments, the overall survival of patients is still unsatisfactory due to the neglected parts of the tumor and inadequate delivery of radiation dose to the primary tumor and lymph nodes. Therefore, it is very

important to determine the effective radiation conditions for better treatment. In this study, we aimed to determine the effects of ^{60}Co γ-ray high dose rate brachytherapy on the survival of HeLa cells with respect to X-ray of 6 MV linear accelerator (EBRT) as a comprehensive radio-biological concept. Furthermore, alterations in RBE values were examined in combined treatments of cisplatin and radiation. According to the results of this study, the RBE rate for γ-radiation from the ^{60}Co brachytherapy was calculated as 1.308. Based on this, it can be concluded that this radiation has a more destructive biological effect than X-ray linear accelerator on cervical cancer cells. Moreover, in combined treatment of cisplatin with X-ray and cisplatin with γ-ray, the RBE obtained for γ-ray was 1.553; which confirmed the hypothesis that the simultaneous treatment of the drug with radiation causes a change in the RBE rate. In 2009, Zhuang et al. examined the effect of ^{125}I radiation on the CL187 human colon cancer cells. The results of RBE evaluation for ^{125}I beam compared to ^{60}Co γ-ray was 1.41, and cell survival showed that ^{60}Co γ-ray was more effective in inhibiting growth, apoptosis induction and cell cycle arrest and ultimately cell death (Zhuang et al., 2009). The potential effectiveness of alternative or new treatments can be evaluated with radiosensitivity parameters from *in vitro* experiments (Veigel et al., 2017). In this study, survival data for HeLa cells were extracted using the LQ survival model. According to the LQ model, the α/β ratios were estimated after EBRT treatment plans. The α/β ratio for the EBRT treatment, and the combined treatment with 1 and 2 μg/ml cisplatin after 2 h were evaluated as 1.057, 3.785 and 4.455 Gy, respectively. Although the α/β ratio for most of the tumors was expected about 10 Gy in clinical practices, some studies based on clinical data and *in vitro* experiments demonstrate that the α/β ratio will be different according to the tumor cell type and tumor stage. In this regard, *in vitro* and clinical studies have shown that the α/β ratio for prostate and breast cancer is much lower than 10 Gy (Brenner and Hall, 1999; Carlson et al., 2004; Qi et al., 2011). These studies opened a new window for further *in vitro* experiments to estimate the radiosensitivity parameters which can be used to predict the clinical outcomes.

Furthermore, a synergistic effect was observed in the combined treatment, including different concentrations of cisplatin with different doses of X-ray. This synergy was considered to be variable depending on the radiation dose, concentration, and duration of cisplatin pre-treatment. The results of combination treatments showed a synergistic effect at all doses of radiation with the concentration of 2 μg/ml pre-treatment at intervals of 1 and 2 h, except for 4 Gy dose with 1 h pre-treatment of cisplatin which had an increasing effect. Consequently, after comparing the time and concentrations of pre-treatments, the concentration of 2 μg/ml and the time of 2 h showed the highest synergy, so this treatment was selected for brachytherapy. Some studies indicated that the mechanism which leads to the synergistic effects of this combination therapy is the non-homologous end joining (NHEJ) pathway (Turchi et al., 2000; Boeckman et al., 2005). When cells are exposed to radiation, various types of DNA damage occur, including double-strand break (DSB) and single-strand break (SSB) as well as damage to nucleobases. DSB repair is performed through the NHEJ pathway, which is also involved in the homologous recombination pathway. The NHEJ pathway in DSB repair requires the activity of several protein complexes such as DNA-PK (DNA-dependent protein kinase), which contains a Ku heterodimer that facilitates the binding of the catalytic portion of two DNA-PK complexes. In various studies, different cell lines with or without DNA-PK were examined. These studies were performed to determine the activity of NHEJ after cell sensitization with cisplatin treatment to investigate the ability of cisplatin to sensitize cells to DNA damage induced by ionizing radiation and the ability to repair DNA double-strand breaks through NHEJ pathway (Chou et al., 1983; Chou, 2006; Chou and Martin, 2005). The exact mechanism of how cisplatin achieves its sensitization activity is not known. However, the *in vitro* analyses support a mechanism involving decreased DSB repair via the NHEJ pathway by cisplatin-DNA lesions inhibiting the kinase activity of DNA-PK through inhibiting the

translocation of the Ku subunits (Turchi et al., 2000).

7. Conclusion

In summary, our results indicated that there was a remarkable synergism in combined treatment of ^{60}Co γ-ray brachytherapy and cisplatin on cervical carcinoma (HeLa) cells. With determination of RBE, it can be concluded that ^{60}Co γ-ray brachytherapy might be more effective than EBRT X-ray in the treatment of cervical cancer; the results also highlight the benefits of chemoradiotherapy in this regard. Further investigations are required to assess the effectiveness of this combination therapy in animal models before it can be translated into the clinic.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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