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Efficiency of modulated and dose rate altered flattening filter free beams in high dose per fraction radiotherapy applications on the survival of prostate cancer cell lines

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ABSTRACT

Background: The radiobiological effect of high dose rate FFF beams on the DU-145 cells was investigated with SBRT plans which have >10 Gy. ***Methods and Materials:*** To compare the radiobiological effect on DU-145 cell line four experiments designed: (1) the constant dose rate of 6 MV and 6 MV FFF with increased dose per pulse (2) the effect of dose per pulse while increasing instantaneous dose rate for 6 MV and 6 MV FFF, (3) the effect of increased average dose rate for 6 MV FFF and (4) the effect of protracted treatment time and modulation of 6 MV FFF beams. The survival fraction was counted with WST. ***Results:*** FF and FFF for 6 MV with same dose rate and treatment time has no effect on cell survival. Significant differences was observed on survival which were irradiated with 6 MV 600 MU/min and 6 MV FFF 1400 MU/min (p=0.024). There was no difference between 6 MV FFF 600 MU/min and 6 MV FFF 1400 MU/min for 10 Gy. The significant survival difference obtained for 20 Gy. The survival percentages for both 10 Gy and 20 Gy with Cyberknife were obtained higher than FFF. ***Conclusion:*** Our *in-vitro* study presented here show that higher dose rate and reduced treatment time might become a crucial factor for SBRT especially which has >10 Gy fraction dose.

Keywords: *Flattening filter free, Cell survival, Radiosurgery, Radiobiology*

INTRODUCTION

New technologies in radiation oncology such as Intensity Modulated Radiotherapy (IMRT) or Volumetric Modulated Arc Therapy (VMAT) have become standard radiotherapy technique that the modulated radiation beams using static or dynamic changing of Multileaf Colimator (MLC), altering dose rate or/and gantry rotation offer significant improvements to dose conformity to the target volume even if inhomogeneous dose distribution is desired within the target volume [1, 2]. Flattening Filter Free (FFF) photon beams by removing flattening filter provide an increase in instantaneous dose rate that poses advantages to radiotherapy. The increased dose per pulse (DPP) through filter removal results in an equivalent increment in the average dose-rate and a potentially similar reduction in overall treatment time. FFF beam options are now commercially available and treatment machines with higher dose-rates are

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being introduced into clinical use. FFF beams allow us to deliver by a factor of 2 to 6 time's faster IMRT/VMAT delivery comparing to standard dose rate in clinical applications [3-6].

The potential radiobiological factors resulting from altering the instantaneous dose-rate include potential changes to sub-lethal damage repair mechanisms, potential synergistic effects and the potential radiobiological effects resulting from changes to the overall treatment time [7,8]. Number of *in-vitro* studies have been undertaken to investigate the implications of changes in dose-rate of the treatment beams resulting from treating with flattening filter free beams [8-12]. Based on recent published data, the effect of changing instantaneous dose rate on cell survival or on cell proliferation is still unclear and must be experimentally studied especially for higher dose (>10Gy) per fraction treatment modalities.

Although few studies [9-11] have reported no differences *in-vitro* cell survival, Lohse *et al.* [12] and recently published data by Hara *et al.* [13] indicated that the FFF beam is more efficient in reducing clonogenic cell survival fraction of glioblastoma cell lines and in increasing antitumor activity of hypoxic cells than FF beams with increased dose rate, respectively.

Stereotactic Radiosurgery (SRS) or Stereotactic Body Radiotherapy (SBRT) modalities are becoming available radiotherapy technologies in routine application at most of the radiotherapy centers. SBRT delivers 40-60 Gy in 1-5 fractions to tumors and SRS irradiates cranial lesions with 18-25 Gy in 1-2 fractions [14]. These impressive clinical efficacies of SRS and SBRT can be explained with induced secondary cell death by causing damages in tumor vasculatures and thereby causing indirect tumor cell death such as immune response addition to direct cells death through DNA double-strand breaks (DSB) [15,16].

However, biological mechanism underlying SBRT and SRS has been elusive. SRS and SBRT typically requires long overall treatment time due to large doses per fraction cause to higher monitor unit (MU) than conventional fractionations. As mentioned above, FFF mode provides shorter treatment times with increasing instantaneous dose rate and may lead to radiobiological advantage with efficient tumor cell killing and more favorable clinical outcomes. Therefore, FFF beams in SRS-SBRT applications were preferred rather than filtered beams. Bewes JM 2008 [15] reported that extended delivery times can increase the cell survival and regional dose rate variation through the tumor that is inherent to IMRT and VMAT, will affect radiation dose efficacy with obtaining synergistic effect which is a potential factor to further increase the therapeutic gain.

Most of the published data to date about whether FFF beams have any radiobiological effect to cancer cell survival was designed with uniform dose distribution of FFF beams. However, in recent work, modulated dose distributions of FFF beams were used for irradiation of tumor cells to assess if the modulated FFF beams have any radiobiological consequences.

In recent work, the radiobiological effect of high dose rate FFF beams on prostate cancer cells line through *in-vitro* experiments with analyzing the metabolically active cells was investigated with highly modulated and altered dose rate non-uniform IMRT/VMAT plans having ≥10 Gy fraction dose.

MATERIALS AND METHODS

Cell culture

The human prostate cancer cell line was used for experimental setup. DU-145 human prostate cancer cell line was provided from the Center for Stem Cell and Gene Therapies Research and Practice, University of Kocaeli. Cell line was cultured in Minimum Essential Medium (MEM) with basal culture medium (Gibco, BRL) supplemented with 10% fetal bovine serum (FBS; Gibco, BRL) and 1% penicillin/ streptomycin (Gibco, BRL), defined as complete culture medium. The cells were cultured at 37°C, 5% CO_2 in a humidified atmosphere, and the medium was refreshed in every two days. After cells reached to 70-80% confluence, cells were detached by 0.25% trypsin-EDTA (Gibco, BRL) and reseeded into the flasks. After irradiation,

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cells were cultured in the same conditions for 5 days.

Experimental setup, treatment planning and irradiation of the cells

The cell culture flasks were placed into the phantom which was filled with rice, designed and implemented for the delivery of treatment plans
to cells *in vitro.* A Computed Tomography (CT) scan was acquired of the setup where flask filled with 1mm cell layer solution placed into the phantom with 1.5 mm slice thickness. All VMAT plans were done on the Eclipse Planning System v11.0 (Varian Medical System, Palo Alto, CA, USA) using 6 MV FF and FFF photons beams with dual full arcs. CyberKnife (CK; Accuray, Sunnyvale, CA, USA) treatment plans were calculated with Multiplan (Accuray, Sunnyvale, CA) treatment planning system. A Varian Trilogy TX linear accelerator and CK systems were used to define the radiobiological effect of altering dose rate, dose per pulse and reducing treatment time on DU-145 prostate cancer cell line with either 10Gy/1fx and 20 Gy/1fx. In practice, the control flasks of each experiment sets were kept in the same environment condition for irradiation of flasks which was done with Trilogy linear accelerator. The experimental fractions were repeated triple per experiment, in case of any unexpected cell culture contamination, poor plating efficiency and definition of standard deviation in the survival ratio of irradiated cell lines. Because of the different plating efficiency, the comparison of cell survival after irradiation Trilogy and CK was evaluated by calculation of survival fraction with standardization of both control groups instead of counting number of survival cells.

Gross Tumor Volume (GTV) was defined using CT slices of phantom which includes outline of the flask filled with 1 mm cell layer solution. Then, Planning Target Volume (PTV) was created by adding 5 mm margin to GTV to ensure that flask (GTV) was covered by 100% of prescribed dose against any possible setup error during irradiation. Treatment plans of VMAT were created with dual arcs to deliver 10 and 20 Gy per fraction. The radiobiological effect of FFF beams was investigated with four different experimental set ups for both 10 Gy and 20 Gy single fraction doses where the effect of increased DPP, increased dose rate, altering average dose rate and protracted treatment time were investigated as explained in detail below. Table 1 summarized the flow chart of all experimental sessions. During the VMAT planning process as first experiments for 10 and 20 Gy dose in single fraction, an approximately average dose rate which is 560 MU/min for 6 MV and 600 MU/min for 6 MV FFF beams were achieved while increasing dose per pulse value at 6 MV FFF beam. The aim of the first experimental setup with keeping average dose rate constant for both 6 MV and 6 MV FFF beams while increasing dose per pulse is to identify the effect of high modulation and variation of dose per pulse with FFF beams. As second experiments, the cells were also irradiated with 6 MV FFF beams having increased instantaneous dose rate that is up to 1400 MU/min while keeping dose per pulse value constant. A variation of the average dose rate was also investigated by analyzing cell survival which were irradiated with 600 and 1400 MU/min for 6 MV FFF beam for 10 and 20 Gy dose as the third experiment.

The fourth experiment was done with CK irradiation of cells. CK treatment plans were prepared with the same conditions with LINAC irradiation experimental set-up of the phantom for doses of 10 and 20 Gy to evaluate effect of protracted treatment time. The plans were utilized with 60 mm collimator and constant dose rate of 800 MU/min therefore we considered the cells in the flasks were treated synchronously during beam on time. The prescription line isodose was 80%. The irradiation details can be seen in table 1.

Quality control of 6 MV plans were evaluated with portal dosimetry system by comparison of the cumulative result of the delivered plans with the dose distribution of the calculated plans considering the percentage of passing rate criteria of gamma value 2%/2 mm. Because the portal dosimerty system was not appropriate to measure FFF beams, the dose accuracy of 6 MV FFF beam's plan was evaluated with 2D (2

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Dimension) ionization chamber array (729 Array Dosimetry, PTW, Freiburg, Germany) system with the same acceptance criteria used for 6 MV accuracy. Furthermore, the output of the linear accelerator for each photon energy at nominal dose rate (600 MU/min) was checked prior to the irradiation of cells using ion chamber according to TRS 398 (17). Dosimetry and accuracy of the CK plans were checked by using ghaphcromic films inserted into phantom by following the E2E test procedure for CK system.

Table 1. Properties of each experiment including dose rate, Monitor Unit (MU), delivery time and dose. Plans named A1 and A2 represent experiments of 6 MV with constant rate for 10 and 20 Gy doses, respectively. Plans named B1 and C1 represent experiments of 6 MV FFF beams for 10 Gy with increased dose rate for 600 MU/min and 1400 MU/min, respectively. Plans named B2 and C2 represent experiments of 6 MV FFF beams for 20 Gy with increased dose rate for 600 MU/min and 1400 MU/min, respectively. Plans named D1 and D2 represent CK irradiations for 10 and 20 Gy doses, respectively.

Plan Name	Technique	Nominal Dose Rate (MU min^{-1})	Dose (Gy)	Delivery Time (mm:ss)
6X_10 (A1)	*Dual Arc VMAT*	560	10	4 min 12 s
2. 6X_FFF_10	Dual Arc	600	10	4 min 9 s
(B1)	VMAT			
3. 6X_FFF_10_14	*Dual Arc*	1400	10	2 min 49 s
(C1)	VMAT			
4. 6X_20	Dual Arc	560	20	9 min 13 s
(A2) 5. 6X_FFF_20 (B2)	VMAT Dual Arc VMAT	600	20	6 min 17 s
6. 6X_FFF_20_14 (C2)	Dual Arc VMAT	1400	20	3 min 57 s
7. 6X_CK_8_10 (D1)	Conformal (6 cm collimator)	800	10	18 min
8. 6X_CK_8_20 (D2)	Conformal (6 cm collimator)	800	20	24 min 45 s

Quantification of viable cells after radiotherapy

The viability of irradiated cells were examined by the WST-1assay test. Following the culture, the medium was replaced with MEM basal medium with 10% WST-1 reagent (Roche, Mannheim, Germany). After incubation for 4 hours, the absorbance at 490 nm was measured by a spectrophotometer (VersaMax, Molecular Devices,CA, USA). The results were normalized by using the fresh culture media with containing 10% WST-1 reagent as a blank.

Statistical analyses

Three replicates of each different dose rate and doses experimental sessions were counted with WST technique to assess the viability fraction of human prostate cancer DU-145 cell lines. The data for all sessions were presented with ± standard deviation. Statistically significance analyses was done by using the Student's t-test method and if p value <0.05 was accepted as statistically significant.

RESULTS

Basically four scenarios were explored for 10 Gy and 20 Gy doses: (1) the effect of constant dose rate of highly modulated 6 MV and 6 MV FFF beams with increased dose per pulse, (2) the effect of dose per pulse while increasing instantaneous dose rate for highly modulated 6 MV and 6 MV FFF beams, (3) the effect of increased average dose rate for 6 MV FFF beams and (4) the effect of protracted treatment time with modulated 6 MV FFF beams. We compared the number of proliferating of DU-145 prostate cancer cells after irradiation with modulated 6 MV and 6 MV FFF energy radiations which have 600 cGy, 800 cGy for CK and 1400 cGy dose rates to evaluate radiobiological effectiveness of FFF

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beams for dose rate, dose per pulse and shorter treatment time for high dose per single fraction dose at 10 and 20 Gy. The dose distributions of the created treatment plans for FF and FFF beams with dual arc and CK were presented in figure 1.

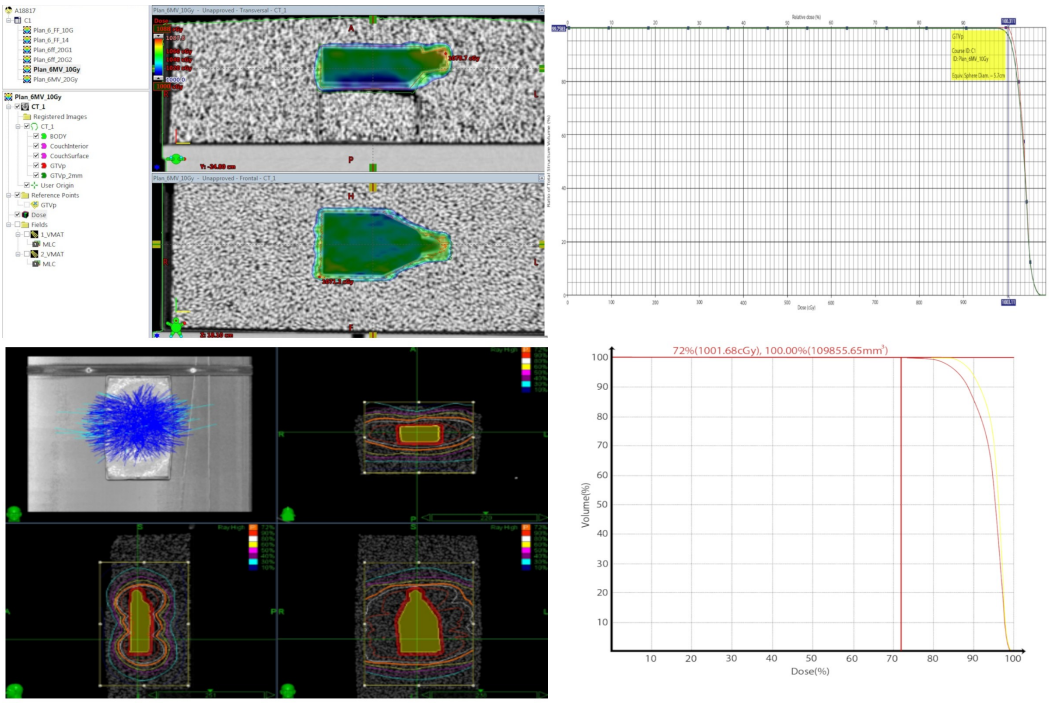

Figure 1. The dose distributions of the VMAT (top) and CK (bottom) plans. As it can be seen that the 95% isodose line fully encapsulated the PTV includes entire flask for VMAT plans and 80% isodose level was selected for prescription doses at CK plans.

The active cell fraction of irradiated DU-145 prostate cancer cells with highly modulated 6 MV and 6 MV FFF beams of constant dose rate while changing dose per pulse as a result of the first experiment presented in figure 2a and 2c for 10 and 20 Gy doses, respectively. Figure 2c represent also the effect of increased dose rate to 1400 MU/min for 20Gy dose. After irradiation with 6 MV and 6 MV FFFF beams for both 10 (figure 2a) and 20 Gy (figure 2c) single doses which have the same average dose rate of 600 cGy/min and different pulse repetition frequencies have resulted with no statistical differences on cell numbers (p=0.062088 and p=0.999788). The differences of metabolically active cells for 10 Gy and 20 Gy were 1.29% and %4.7, respectively.

The effect of the increased dose rate keeping approximately same dose per pulse for 10 Gy dose in single fraction are presented in figure 2b as a result of the second experiment. Statistically significant differences were observed for survival status of cells which were irradiated with increased instantaneous dose rate and keeping almost constant dose per pulse for both 10 and 20 Gy a single dose 6 MV 600 MU/min and 6 MV FFF 1400 MU/min (p=0.0249 and p=0.0207) as seen in figure 2b and 2c, respectively. The fold change in the cell proliferation was 0.32 which was almost 3-fold decreased at 10 Gy for 6 MV FFF 1400 MU/min and 0.48 at 20 Gy for 6 MV FFF 1400 MU/min versus 0.49 at 10 Gy for 6 MV 600 MU/min and 0.61 at 20 Gy for 6 MV 600 MU/min. Although there was no difference between the cell numbers which were irradiated with 6 MV FFF 600 MU/min and 6 MV FFF 1400 MU/min having approximately same dose per pulse for 10 Gy (p=0.632), the statistically significant difference in cell proliferation was obtained for 20 Gy single fraction (p=0.028). The decrease in the cell proliferation of these irradiation setups were 0.36 and 0.32 at 10 Gy versus 0.56 and 0.48 at 20 Gy.

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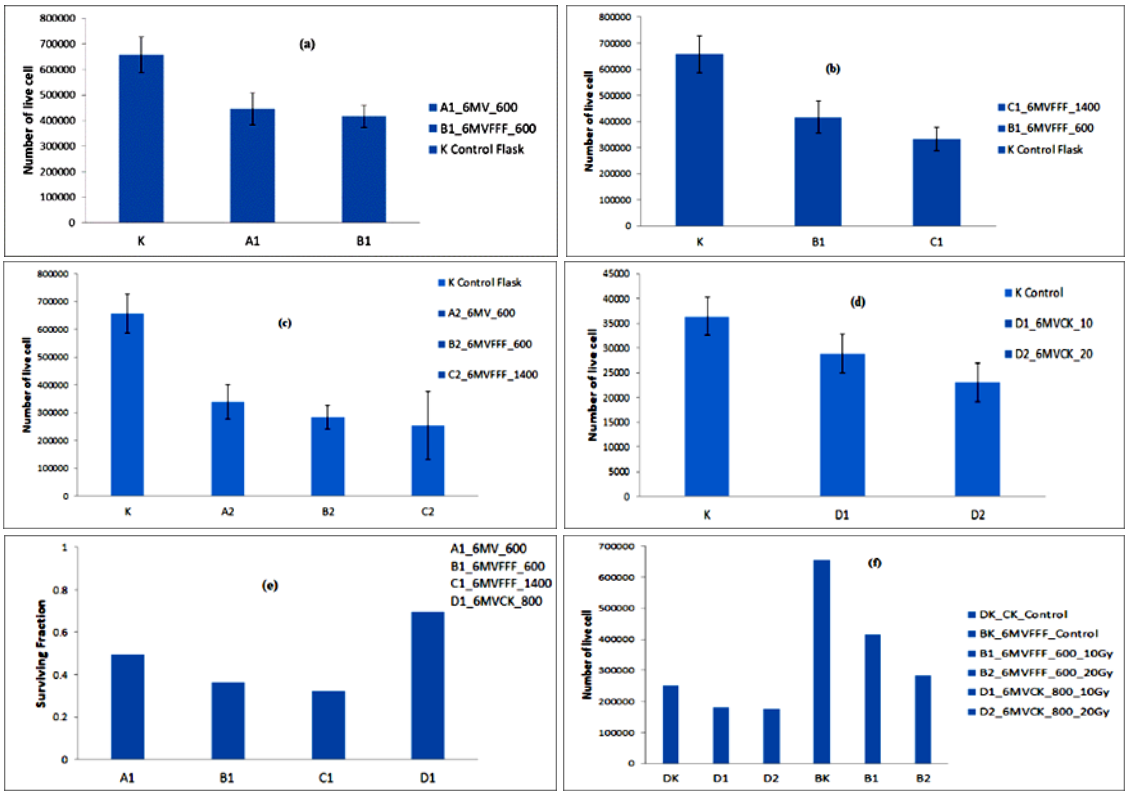

Figure 2. Graph **(a)** presents live cell number after modulated flattening filter free (FFF) and flattening filter (FF) irradiation for 10 Gy dose at 600 cGy/MU dose rate with varying dose per pulse. Graph **(b)** presents cell survival after 10 Gy exposure with changing dose rate for 6 MV FFF beams. Graph **(c)** presents the result of cell survival fraction after increased per pulse and dose rate for 20 Gy dose. Graph **(d)** presents the result of cell survival fraction after CK irradiation for 10 and 20 Gy doses. Graph **(e)** shows survival fraction of cells after irradiation with different average dose rate FFF beams and 6 MV with 800 cGy dose rate for 10 Gy dose. Graph **(f)** shows the difference of survival cell reduction between CK and 6 MV FFF beam for 10 and 20 Gy doses.

Figure 2d shows also the metabolically active cells after irradiation for the fourth experiment set up condition, which were irradiated with CK with constant dose rate (800 MU/min) for total doses of 10 and 20 Gy to evaluate radiobiological effect of extended treatment time and non-modulated 6 MV photons. Each column includes the mean ± SD of three independent irradiations of flask for each experimental setup.

The survival difference between 10 and 20 Gy doses irradiation with CK was compared with 6 MV FFF beam which has 600 cGy dose rate for same doses that treatment times for each irradiation set up were 4 min 9s, 6 min 17s for 6 MV FFF, 18 min and 24 min 48s for CK, respectively. The survival fraction ratio differences for 10 and 20 Gy doses in CK irradiations were found smaller than 6 MV FFF beam with 600 cGy dose rate which has shorter treatment time comparing to CK irradiations with the differences of 5.1% and 30% for CK and 6 MV FFF irradiations, respectively. Figure 2(e) presents the result of the survival fraction of the cells irradiated with 6 MV at 600 MU/min, 6 MV FFF at 600 MU/min, 6 MV FFF at 1400 MU/min and CK at 800 MU/min for 10 Gy single fraction dose to evaluate radiobiological effect of extended treatment time and non-modulated 6 MV photons. The comparison of cell survival fraction between unmodulated 6 MV CK and modulated 6 MVV FFF beam irradiation for 800 cGy and 600 cGy dose rate was expressed as a percentage due to the initial cell numbers of control flask in CK groups which thought to be affected by environmental conditions were smaller than other groups. The comparison of proliferation rates between unmodulated 6 MV CK and modulated 6 MV FFF beams was calculated after standardization of results for CK

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and 6 MV FFF irradiations and the result of this experiment was presented in figure 2(f). The enhanced cell survival was observed for cell irradiated with CK as can be seen on figure 2(e) that the survival was 0.7886 at 10 Gy and 0.7455 at 20 Gy while the survival of flattened and unflatten beams with 600 MU/min and 1400 MU/min dose rate were 0.5054 and 0.6344 and 0.3859 and 0.4329, respectively. The survival cell number of CK and 6 MV FFF beam for 10 and 20 Gy and control groups of both experimental set up was shown in Figure 2(f).

DISCUSSION

While the radiobiological effect of SRS and SBRT has not been fully known, the radiological results of the FFF beams are also added to this unknown which is as a matter of debate needing further investigation. The dose rate of beam delivery can be modulated in two manners: by varying the pulse repetition frequency (PRF) or by changing the dose per pulse (DPP) through filter removal. In this study, the effect of almost two times increased DPP was evaluated at both 10 Gy and 20 Gy with constant dose rate of 600 MU/min using double arc VMAT plans 6 MV and 6 MV FFF beams resulting with a potentially similar cell survival and no statistical significance. The results presented in this study showed that the increased instantaneous dose rate using FFF beam have more radiobiological effectiveness than increased DPP value for DU-145 human prostate cancer cell line which were irradiated using 6 MV conventional beam and 6 MV FFF beam with 600 and 1400 MU/min dose rate for high single fraction doses of 10 and 20 Gy. The high dose rate effect was more significant at dose of 20 Gy that is clinically more relevant dose for SRS/SBRT applications. Although the potential radiobiological factors resulting from changing the instantaneous dose-rate include the potential changes to sub-lethal damage repair mechanisms, the potential synergistic effects and the potential radiobiological effects resulting from changes to the overall treatment time [18], there is also the possibility that these high instantaneous dose rates can result in synergistic effects where the high photon fluency can lead to collective interaction behavior and enhanced radiobiological damage [15,19,20].

Our experiments in this study resulted with increased dose rates in FFF beams with stronger radiobiological effects especially at 20 Gy single fraction dose on DU-145 cell line by obtaining decreased cell survival. This observed radiobiological effect caused by different potential mechanisms as mentioned above should be clarified by investigations of damages on different cell lines. With this purpose, we aim to do a future work to explore the radiobiological implications of FFF beams with instantaneous dose rate and modulation by using different tumor and normal cell lines which have different radiosensitivity, cell cycle, doubling time and different sublethal repair time. The further investigation of the radiobiological effects of FFF beams was done with A-549 lung carcinoma and H-EMC-SS human chondrosarcoma cell lines by repeating the same experimental set up with this study.

We also investigated the effect of protracting delivery time using intermittent irradiation produced by CK which has approximately 5 and 3 folds more for 10 and 20 Gy doses, respectively. Although irradiation condition of cell lines were different from the experiments with LINAC irradiation, the comparison of the survival of cells irradiated with CK and FFF beams was presented as a percentage that resulted with higher survival fraction achieved at cells protractedly irradiated with CK. The irradiation of flask with CK was done in a different institute and the survival of cells in flask was affected by movement condition which can be assumed as a limitation of this study.

Although the protracted treatment time effect was investigated by different authors [7, 21-25] in terms of radiobiological efficiency of IMRT versus VMAT and FF versus FFF beam, due to the absence of comparison study of CK treatment and FFF beam which has very shortened treatment time according to CK treatment, the radiobiological differences of FFF and CK treatment need to be clarified especially for lung and liver SBRTs. The future direction of

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this study was designed to compare radiobiological result of FFF and CK treatments with different radiosensitive tumor cell lines.

In this study, the WST1 assay was used to determine the number of viable cells and to assess the effect of irradiation on cells after a short period of in vitro culture. Moreover, MTT tetrazolium salt colorimetric assay and Colony-Forming-Unit (CFU) are two different methods, which are generally used for this purpose. Rather the determination of the clonogenic potential of viable cells by CFU, the effect of various irradiation parameters on cells was quantified by measuring the metabolic activity using WST1 assay. The culture of cells for longer time period was avoided to minimize any variable (i.e. culture media, pH, temperature and cell specific factors. The paper by Guertler *et al.* 2011 [26] validated the usefulness of WST-1 assay in screening for radiation-sensitive cells. WST-1 assay is more sensitive to detect small differences on cell viability. Therefore, the irradiation doses were evaluated by WST-1 assay rather CFU. Similar papers also used this method to assess the effect of various irradiation doses [27, 28]. The cell sensitivity and metabolic activity against the varying radiation doses was evaluated by WST1 to show the cancer cell survival after radiation. The colonogenic capacity of the cells is directly related with the tumor formation, but the therapy effectiveness can also be correlated with the reduction of cell growth, which is measured by MTT or WST-1 [29].

The metabolic activity also affects the cancer cell sensitivity to subsequent therapies. As the WST1 assay is based on the reduction of the tetrazolium dye WST1 to water-soluble formazan by nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes, the decrease in the cellular energy metabolism would directly affects the other cellular events, like as proliferation and migration. As the radioresistance is associated with the alterations in the cell metabolism and the main cause of radiotherapy failure is cellular radioresistance, conferred via glycolytic or mitochondrial metabolic changes [30]. Therefore, the evaluation of metabolically active cells can also show the efficiency of the radiation therapy and WST1 assay can partially substitute the clonogenic assay in order to determine survival of irradiated tumor cells [31].

Recently published data on the radiobiological effect of FFF beams with high dose rates show discrepancies because of the heterogeneity of cell lines and experimental settings. Lohse 2011 [12] reported differences between cell survival in the T98H and the U87 cell lines in favor of the higher instantaneous dose rate while the average dose rate and treatment times of 10 MV beams were constant. Our findings are in agreement with Lohse 2011 [12] especially at 20 Gy single fraction dose. Sarojini 2015 [32] also observed similar result with our findings that although the cell survival differences at up to 8 Gy doses were not as high as at low dose for cell lines except WC00060 cell line, the significantly higher apoptosis at a dose rate 2400 MU/min was observed in melanoma cell lines than a dose rate 400 MU/min with low total doses.

Furthermore, our results agree with recently published data from Hara *et al.* [13] that they reported significant dose rate-dependent difference in antitumor activity in hypoxic cells, when FFF beams are used. They also reported greater DNA damage and reduced cell proliferation at increased dose rate in hypoxic cells. Whereas King 2013 [10] published a report resulting no differences between survival fraction of 6 MV and 6 MV FFF beams up to 8 Gy single fraction doses with the unmodulated beams, unlike this study, our experimental irradiation were designed with modulated beams with 20 Gy per fraction acute dose using human DU-145 prostate cancer cell line. Sorenson 2011 [9] and Verbakel 2013 [33] found no change in cell survival as a result of increasing the instantaneous dose rate and shorter treatment time. Previous in vitro studies have investigated the biological effect of the high dose rate of FFF beams via using either a compensator or small field size to use homogeneous part of profile that the profile of under 5×5 cm2 field sizes of FFF beams have similar behavior with flattened beams by

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inverse square rule [9, 11].

Recently Verbakel 2013 [33] used dynamic IMRT technique with static gantry angle with maximum 10 Gy single fraction dose. In case of Verbakel WF 2013 [33] IMRT was used as a delivery method with dynamic MLC whereas we have used VMAT in this study for obtaining reduced treatment time which is a specific type of IMRT where the MLC and dose rate can be altered during gantry rotation to taking into consideration effect of modulation, and potential synergistic effects between cells using up to 20 Gy single fraction dose. As shown previous studies in terms of compression IMRT and VMAT in clinical plans that VMAT can provide highly conformal plans while reducing treatment time compared to IMRT [34, 35].

A number of *in-vitro* studies have showed that increased cell survival which is the evidence of potential increase in the radiobiological effectiveness in clinical outcomes was observed with protracted delivery time associated with IMRT [21-24]. The associated reduction in delivery time could allow for increased cell death through a reduction in sub lethal damage repair mechanism.

CK delivery method was used to simulate 6 MV FFF beam with unmodulated, protracted delivery time with variable dose rate that the percent of viable cell was higher than VMAT plans in this study. This result can also be explained with fast tumor cell repair of sub-lethal damage because of protracted delivery time and/or the by-stander effect [25] could be an alternative effect to increase cell survival with the communication of cells between irradiated with small dose and nearby non-irradiated tumor cell. However survival differences in this study cannot be explained only by-stander effect, because even if modulated high dose rate fields were used to irradiate all flasks, the defined GTV which includes tumor cells were covered by the same dose levels. Our result is also consistent with Yang 2009 [36] that the cell survival can be decreased when the delivery method is continuous irradiation instead of segmented irradiation or irradiation with interval between beam-on steps similar to CK delivery method. In treatment using large doses per fraction, the influence of protracted treatment time could be important with late reacting normal tissues being generally more sensitive to the dose rate effect than tumors and early reacting tissues.

Consequently, the result of this study in terms of protracting treatment time are in agreement with the study of Karan 2013 [37] that the implication of faster radiation delivery could result with enhanced cell killing and therefore increased therapeutic gain could be achieved with VMAT delivery technique using high dose rate FFF beam for SRS/SBRT. Even though all of the above mentioned studies in terms of increasing dose rate and dose per pulse effects have no differences on cell survival they concluded that additional studies are necessary to clarify the existing debates on the radiobiological effects of high dose rate FFF beams for large single fraction dose treatment for cancer and normal tissue cells placed out of target cells to identify any possible late complications. This study contributes to the growing number of investigations for the radiobiological effects of varying instantaneous dose rate, by providing the results with increased survival fraction correlated with altered instantaneous dose rate and highly modulated FFF beams, for SRS/SBRT at the dose levels up to 20 Gy.

CONCLUSION

Our study as an *in vitro* verified that modulated FFF beams with increased instantaneous dose rate would alter cell survival especially in high dose single fraction such as SBRT/SRS irradiation. According to our results using DU-145 human cancer line, protracted treatment may cause to reduced local control even at high dose per fraction irradiation.

As a conclusion, modulated FFF beams with higher instantaneous dose rate for high dose per fraction irradiation might cause synergistic radiation effects that should be investigated with different cell tumor lines even with normal tissue cell lines.

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