Impact of Prolonged Fraction Delivery Time Modelling Stereotactic Body Radiation Therapy with High Dose Hypofractionation on the Killing of Cultured ACHN Renal Cell Carcinoma Cell Line

Khorramizadeh M.[1], Saberi A.[2]*, Tahmasebi–birgani M.[1], Shokrani P.[3], Amouhedari A.[4]

[1]Department of Medical Physics, Faculty of Medicine, Dezful University of Medical Sciences, Dezful, Iran
[2]Department of Medical Genetics, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[3]Department of Medical Physics and Medical Engineering, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran
[4]Department of Radiation Oncology, Milad Hospital, Isfahan, Iran

ABSTRACT

Introduction: Stereotactic body radiotherapy delivers hypofractionated irradiation with high dose per fraction through complex treatment techniques. The increased complexity leads to longer dose delivery times for each fraction. The purpose of this study is to investigate the impact of prolonged fraction delivery time with high-dose hypofractionation on the killing of cultured ACHN cells.

Methods and Materials: The radiobiological characteristics and repair half-time of human ACHN renal cell carcinoma cell line were studied with clonogenic assays. A total dose of 20 Gy was administered in 1, 2 or 3 fractions over 15, 30 or 45 min to investigate the biological effectiveness of radiation delivery time and hypofractionation. Cell cycle and apoptosis analysis was performed after 3-fraction irradiation over 30 and 45 min.

Results: The α/β and repair half-time were 5.2 Gy and 19 min, respectively. The surviving fractions increased with increase in the fraction delivery time and decreased more pronouncedly with increase in the fraction number over a treatment period of 30 to 45 min. With increase in the total radiation time to 30 and 45 min, it was found that with the same total dose, 2- and 3-fraction irradiation led to more cell killing than 1-fraction irradiation. 3-fraction radiation induced G2/M arrest, and the percentage of apoptotic cells decreased when the fraction delivery time increased from 30 min to 45 min.

Conclusion: Our findings revealed that sublethal damage repair and redistribution of the cell cycle were predominant factors affecting cell response in the prolonged and hypofractionated irradiation regimes, respectively.

Keywords

Hypofractionation, Prolonged Fraction Delivery Time, Renal Cell Carcinoma, Stereotactic Body Radiotherapy, Sublethal Damage Repair

Introduction

Clear cell renal cell carcinoma (ccRCC) is the most common and apparently the most aggressive RCC subtype. About 70-80% of kidney cancers are made up of clear cells [1]. ACHN, CAKI-1 and A498 cell lines are ccRCC type [2, 3]. The genetic association

*Corresponding author:
A. Saberi
Department of Medical Genetics, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
E-mail: ahsaberi70@hotmail.com

Received: 25 May 2016
Accepted: 8 September 2016

of RCC is associated with Von Hippel-Lindau (VHL) gene with chromosomal loss in 3p25-26. Mutations of VHL gene were also found in 60–80% of ccRCC [4]. ACHN cell line was initiated in November, 1979 from the malignant pleural effusion of a 22-year-old Caucasian male with widely metastatic renal adenocarcinoma [5].

For at least three decades, renal cell carcinoma (RCC) has been considered to be a relatively "radioresistant" tumor. In clinical setting, it typically refers to tumors that are poorly controlled with conventional radiotherapy schedules [6]. Because of the dose-limiting critical normal structures such as the bowels, spinal cord and the remaining kidney, surrounding the tumor, high dose radiation cannot be delivered to the tumor site in conventional radiotherapy [7].

Stereotactic body radiotherapy (SBRT) has been used as an alternative to surgery for RCC [8-10]. SBRT optimizes the physical dose distribution of radiotherapy by enhancing local tumor control and reducing radiation-induced toxicities. This method is especially useful for treating tumors (e.g. RCC) which are surrounded by many critical structures [11]. Using a linear accelerator, stereo¬tactic irradiation generally uses multiple arc or fixed-portal photon beams, and a few minutes of beam-off time is usually necessary for setting the respective arcs or ports. Consequently, a markedly longer time, ranging from 5 min to 1 h or even longer, is required for one treatment session [12, 13].

In the 1960s, Elkind and colleagues reported that the survival of cultured mammalian cells irradiated using intervals between two radiation doses increased. This phenomenon is attributable to the repair of sublethal damage [14, 15].

From a radiobiologic point of view, sublethal damage repair (SLDR) takes place not only between the fractions, but also during the irradiation [16]. When total treatment time is extended, tumor cell killing tends to decrease because of SLDR processes that occur during the interfraction period [17]. The effect of SLDR on treatment outcome is more significant for tumors with a low α/β ratio and short repair half-time [18]. The linear-quadratic (LQ) model is generally used for calculating radiotherapeutic isoeffect doses for different fractionated radiotherapy schedules. The LQ model encompasses two components α and β which characterize non-repairable and repairable damage, respectively. This model assumes that the biological outcome of irradiation is directly proportional to total dose and fraction size; ratio of α and β (α/β) indicates the sensitivity of tissues to different fraction sizes [19]. Many studies have addressed the impact of prolonged fraction delivery time on tumor cell killing with a 2 Gy dose per fraction or a low dose per subfraction [20-22]. Zheng et al. investigated this effect with variable total dose delivery protocols in the IMRT technique [17, 23]. In the systematic review, Kothari et al. [24] analyzed outcomes and toxicity of stereotactic radiotherapy in metastatic RCC. They reported that Stereotactic radiotherapy is associated with excellent local control and low rates of toxicity for intracranial and extracranial metastatic RCC. They gust used the α/β of two human RCC cell lines, Caki-1 and A498 (6.9 and 2.6, respectively) to evaluate single dose regimens or hypofractionated regimens. ACHN or other cell lines were not taken into account in their study. However, only a few studies have investigated the effect of treatment time in SBRT on the tumor response [12, 13]. The purpose of this study was to investigate the radiobiological effect of prolonged fraction delivery time on the survival of human renal cell carcinoma cell line, ACHN, by using high dose hypofractional regimens with a constant total dose delivery protocol.

Material and Methods

Cell Culture

The ACHN renal cell carcinoma cell line purchased from the Iranian Biological Resource Center was maintained in Minimum Essential medium in Earl's BSS supplemented with 10% heat inactived FBS, 1% non-essential amino acids, 1 mM sodium pyruvate, 2 mM l- Glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin. The cell line was incubated at 37° C in 5% CO2 in air; when cells reached approximately 80% confluency, they were trypsinized with 0.25% trypsin-EDTA and then subcultured.

Irradiation Conditions

All irradiations were carried out using a 6 MV photon beam produced by a clinical linear accelerator (ONCOR; Siemens Company, Germany). A six-well plate containing ACHN cells was placed in a phantom composed of a rectangular block of solid water with a plate-sized cavity at the center. A 5cm thick solid water slab was placed at the bottom of the phantom to ensure the full backscatter condition. A 1.5 cm thick solid water slab was placed on the top of the plate to serve as a build-up material for the 6 MV beam (Figure 1). The plates were irradiated using 20 × 20 cm^2 field size and a dose rate of 3 Gy/min. In vivo diode (QED; Sun Nuclear Company, United State America) dose radiation measurement was performed to ensure the accuracy of delivered dose within ±2%.

Evaluation of Radiobiological Parameters (α/β Ratio and Half Time of SLDR ($T_{1/2}$))

Cells were harvested from a stock culture and plated with agar at densities of 200 cells (0 Gy), 800 cells (2 Gy), 2000 cells (4Gy), 4000 cells (6 Gy), 8000 cells (8 Gy) and 15000 cells (10 Gy) into 6-well plates to establish a survival curve and thus evaluate. Then, doses of 0, 2, 4, 6, 8 and 10 Gy were administered as a single fraction to establish a survival curve and thus evaluate "alpha/beta ratio". A total dose of 8 Gy was administered in two fractions with an interfraction interval of 0.25–4 h to determine $T_{1/2}$. After irradiation, all plates were incubated together overnight at 37oC in order to allow time for potential lethal damage repair. After 24 h, cells in each well were trypsinized and counted using a hemocytometer; then, they were investigated with the soft agar

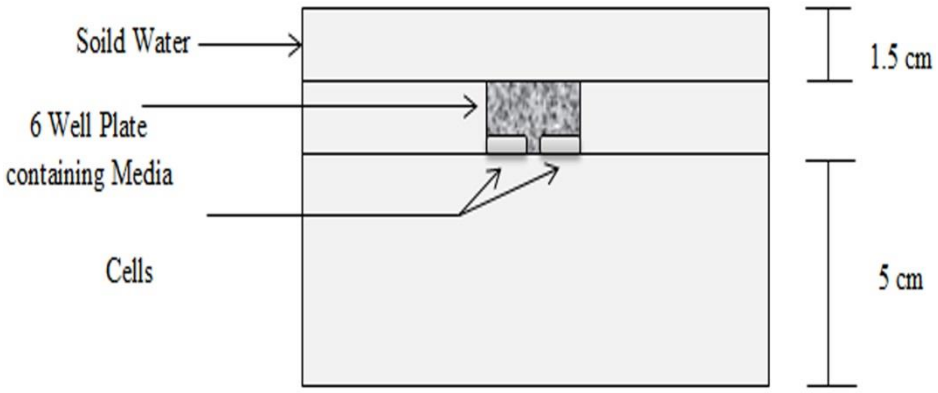

Figure 1: Schematic cross-section of the cell irradiation phantom

colony formation assay.

Impact of Fraction Delivery Time on Cell Killing

To compare the effectiveness of cell killing of fraction delivery time modelling SBRT, the cells were irradiated with a total dose of 20 Gy in 1, 2 and 3 fractions with six equal sub-fraction and inter-subfraction intervals of 3, 6 and 9 min to simulate fraction duration times (FDTs) of 15, 30 or 45 min (Figure 2), respectively. Similar to the clinical dose-time-fractionation pattern, one fraction was administered per day.

Soft Agar Colony Formation Assay

Soft agar colony formation assay was utilized to acquire the dose-survival curves of ACHN and to determine the effect of irradiation modelling SBRT with a fraction delivery time of 15, 30 and 45 min. Cells were plated on top of 1% bottom agar in growth medium and overlaid with 0.3% top agar in growth medium. Cells were fed 2 mL of growth medium every 3–4 days for 4 weeks. Colonies (containing ≥50 cells) were stained with a 0.05% aqueous solution of crystal violet and counted.

For the unirradiated control, 200 cells were inoculated into a six-well plate and allowed to grow. The plating efficiency (PE) was determined as the percentage of the number of colonies observed to the number of cells seeded. The surviving fraction (SF) is the ratio of the number of colonies produced to the number of cells plated, with a correction necessary for PE: Surviving fraction = colonies counted/ [cells seeded × (PE/100)]. Measurement of the cell survival fraction was repeated three times, and the survival data were fitted to the LQ model.

LQ Model

The LQ model has been widely used to fit both the in-vitro experimental data and clinical data [25, 26]. In this model, the surviving fraction S of cells irradiated to a total dose D is given by

$$S= \exp (-\alpha D-\beta GD^2) \qquad (1)$$

where α and β characterize intrinsic radiosensitivity, and G is the dose protraction fac-

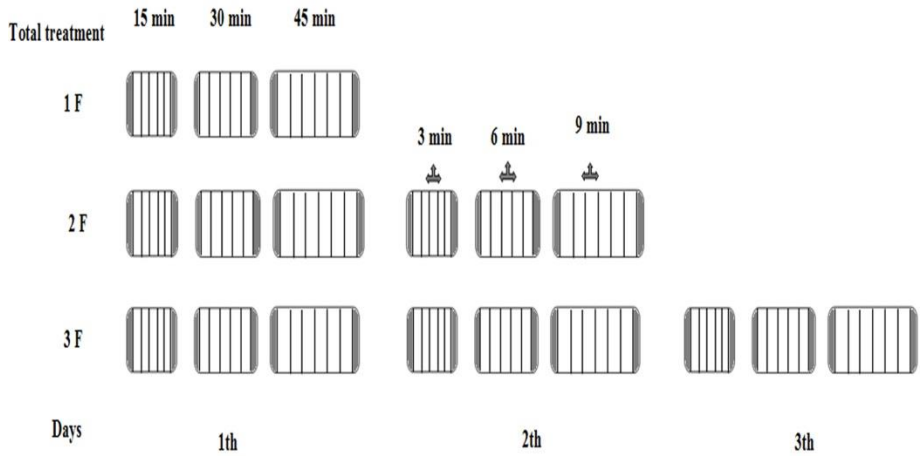

Figure 2: Schematic diagram of 15, 30, and 45 min radiation fractionation schedules with 3 , 6 , and 9 min inter-subfraction intervals, respectively.
F= Fraction

tor. For split-dose exposure with two equal fractions, G has the form of:

$$G = \frac{1}{\mu T_f}\left[1 - \frac{1}{\mu T_f}\left(1 - e^{-\mu T_f}\right) + \frac{e^{-\mu T_i}}{2\mu T_f}\left(1 - e^{-\mu T_f}\right)^2\right] \quad (2)$$

where μ is the repair rate of tumor cells (μ = ln2/T_r, where T_r is the repair half-time), T_f is the dose delivery time and T_i is the time interval between the two fractions.

Cell Cycle Analysis

Twenty-four hours after exposure, cells with 3-fraction radiation for 30 and 45 min were collected and washed with cold PBS; then, they were kept in precooled 70% ethanol at -20°C for fixation overnight. The cells were stained with 0.1% (v/v) Triton X-100 in PBS, 0. 2 mg/ml RNase-free DNase A and 20 μl of 1 mg/ml PI. After an incubation time of 30 min, the cell-cycle distribution was measured with a FAC Scan Flow Cytometer (Becton–Dickinson).

Apoptosis Assay

Twenty-four hours after exposure, cells with 3-fraction radiation for 30 and 45 min were harvested with trypsin, washed twice with cold PBS and then re-suspended in binding buffer. After that, cells were stained with Annexin V-FITC and propidium iodide using Annexin V-FITC Apoptosis Detection Kit I. FACS Flow cytometer was used to quantify the percentage of apoptotic cells.

Statistical Analysis

Statistical analyses were carried out using SPSS 20 software package and are presented as means ± SE. One-way ANOVA was used to compare the data among three groups. Student's t-test was used to compare two groups. In all analyses, p value less than 0.05 was considered statistically significant.

Results

Radiobiological Characteristics of ACHN Cells

Figure 3 shows the survival curve measured with single-dose exposure. The irradiation dose ranged from 2 to 10 Gy. The error bars of each point represent the standard deviations calculated from the repetition of experiment 3 times with a dose rate of approximately 3 Gy/min. The measured and estimated surviving fractions of 2 Gy (SF2) were approximately 0.69 and 0.71, respectively. The LQ model was used to fit standard dose-survival curves of ACHN and determine the α/β ratio. The best fit was done with G(t) =1, since the dose was delivered acutely [18]. The low α/β ratio of ACHN cells demonstrated that they might be radiosensitive to treatment using a hypofractionation schedule.

Figure 4 shows the cell surviving fraction as a function of time interval between split doses. The split doses of 4 Gy + 4 Gy were delivered with time intervals of 0, 0.25, 0.5, 0.75, 1, 2, 3 or 4 h to ACHN cells. The curve shows the results of fitting based on Equations 1 and 2.

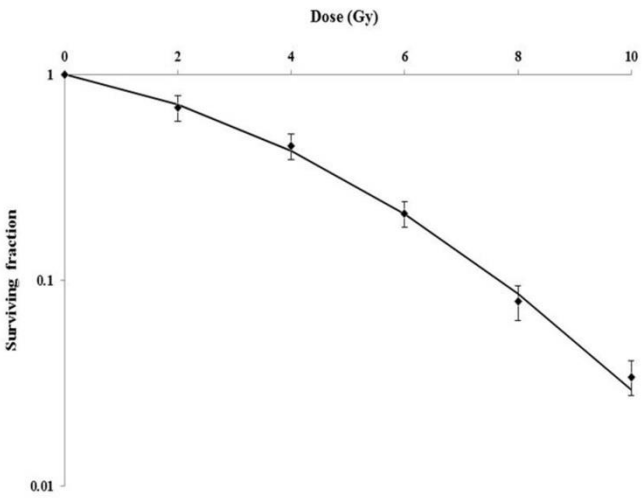

Figure 3: Dose-survival curve of ACHN cells, fitted by the linear-quadratic model (solid curve)

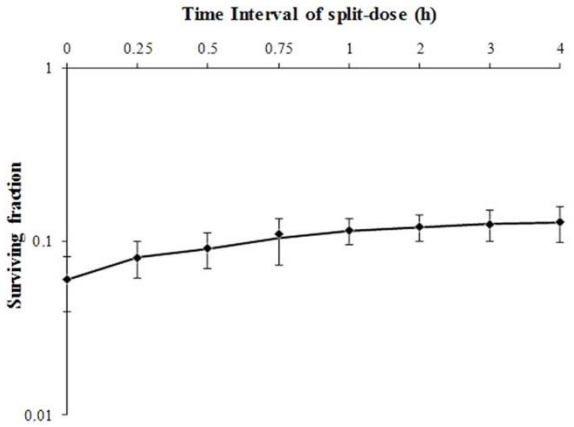

Figure 4: Sublethal repair curve for ACHN cells, using split-dose exposure of 8 Gy and fitted by the linear-quadratic model (solid curve)

The α/β extracted from equation 1 was used in equation 2 to evaluate the half-repair time. The short $T_{1/2}$ (19 min) may be comparable to the fraction dose delivery time for SBRT; consequently, killing of ACHN cells might be affected by the prolonged delivery time.

The radiobiological characteristics of the cell line described with the parameters of the mathematic models are listed in Table 1.

Impact of Fraction Delivery Time on Cell Killing

Surviving fractions of irradiation modelling fractionated SBRT of 20 Gy × 1, 2 and 3 fractions, which were administered in six equal sub-fractions per fraction and delivered within a total fraction delivery time of 15, 30 or 45 min are shown in Figure 5. In all fractionation schedules, the cell irradiated modelling SBRT with longer fraction delivery time led to significantly greater survival than that seen with shorter fraction delivery time ($P < 0.05$).

While no significant differences were noted in the survival of ACHN cells for the different hypofractionation schedules over 15 min ($P > 0.05$), the relative surviving fractions significantly decreased with increase in the fraction number over FDTs of 30 to 45 min ($P < 0.05$).

Effect of Fraction Delivery Time on Cell Cycle Distribution

Flow cytometry analysis (PI stain) was used to determine the effect of FDT on cell cycle distribu¬tion of ACHN cells. The control (a) and 3-fraction radiation over 30 (b) and 45 min (c) groups are shown in Figure 6. The population of cells in G2/M with FDT of 30 and 45 min were 59.02%±3.81% and 59.64%± 3.44, respectively, versus 20.59%±2.32% in the control group. The Fluorescence-activated cell sorting (FACS) results showed that the G2/M population in ACHN cell significantly increased and G0/G1 population decreased following 3-fraction radiation over 30 and 45 min compared with control (P-Value < 0.05). Additionally, no statistically significant difference was detected in the percentage of cells in G2/M among the 3-fraction radiation groups (P-Value > 0.05).

Table 1: Radiobiological characteristics of ACHN described with parameters derived from the dose-survival curves fitted by the linear- quadratic model

cell line	SF_{2read} *	SF_{2est} **	α(Gy^{-1})	β(Gy^{-2})	α/β(Gy)	$T_{1/2}$(min)
ACHN	0.69	0.71	0.12	0.023	5.2	19

* survival fraction of 2Gy (read)

** survival fraction of 2Gy (estimated)

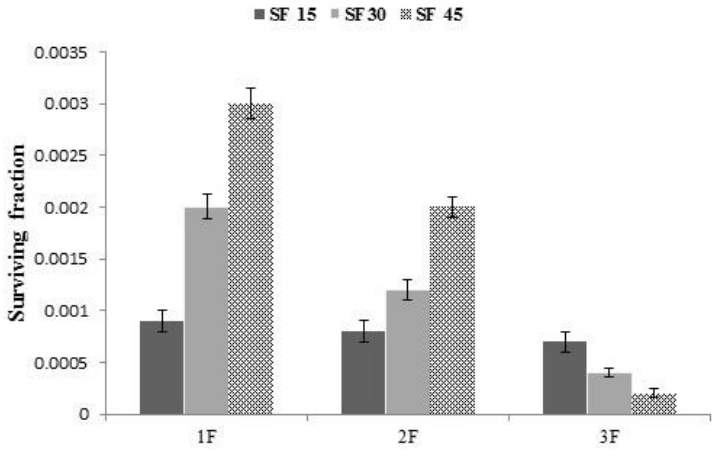

Figure 5: Surviving fraction of ACHN cells after exposure to 20 Gy administered in 1–3 fractions with a total fraction delivery time of 15, 30 or 45 min.
F= Fraction; SF = Survival Fraction

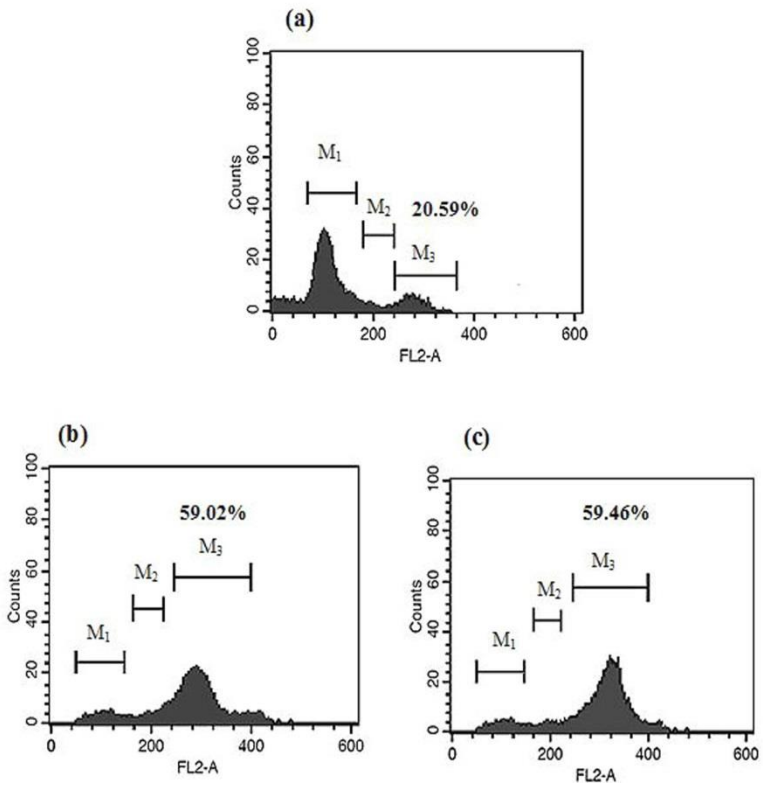

Figure 6: Effect of 3-fraction on the cell cycle distribution of the ACHN cell. (a) Control. 3-fraction (b) over 30 and (c) 45 min.
M1= G1; M2 = S and M3= G2/M

Effect of Fraction Delivery Time on Apoptotic Cell

To study the effect of prolonged fraction delivery time on the apoptosis of ACHN cells, apoptosis of cells was measured using flow cytometry with the Annexin V/PI apoptosis

detection kit. Cells were categorized into the following four populations: Early apoptotic (right bottom), late apoptotic (right top) and necrotic (left top) cells. As shown in Figure 7, the group exposed to a FDT of 30 min showed a significant decrease in the percentage of extent of apoptosis compared to the group with a FDT of 45 min. The percentage of apoptotic cells in the groups with FDT of 30 and 45 min were 76.2 ± 5.4 and 50.7 ± 3.9%, respectively.

Discussion

In this study, an in-vitro experiment using human cell line ACHN was performed to examine the effect of prolonged fraction delivery time in SBRT on cell killing, with a total high dose of 18 Gy in 1, 2 or 3 fractions with six equal sub-fractions per fraction.

For the first time, we found α/β equals 5.3 Gy for ACHN cell line using 6-MV X-ray produced by linear accelerator. ACHN α/β is not representative of all kidney cancers. Ning et al. [27] measured the α/β ratios of two different renal cell carcinoma cell lines; A496 and CAKI-1 irradiated with Cs137 in vitro and found them to be 2.6 and 6.9 Gy, respectively.

Increased cell survival was seen for all fractionation schemes when the total treatment time increased from 15 min to 45 min. Generally, when a dose of radiation spreads over a period, SLDR considerably affects the response of irradiated cells, and increase in

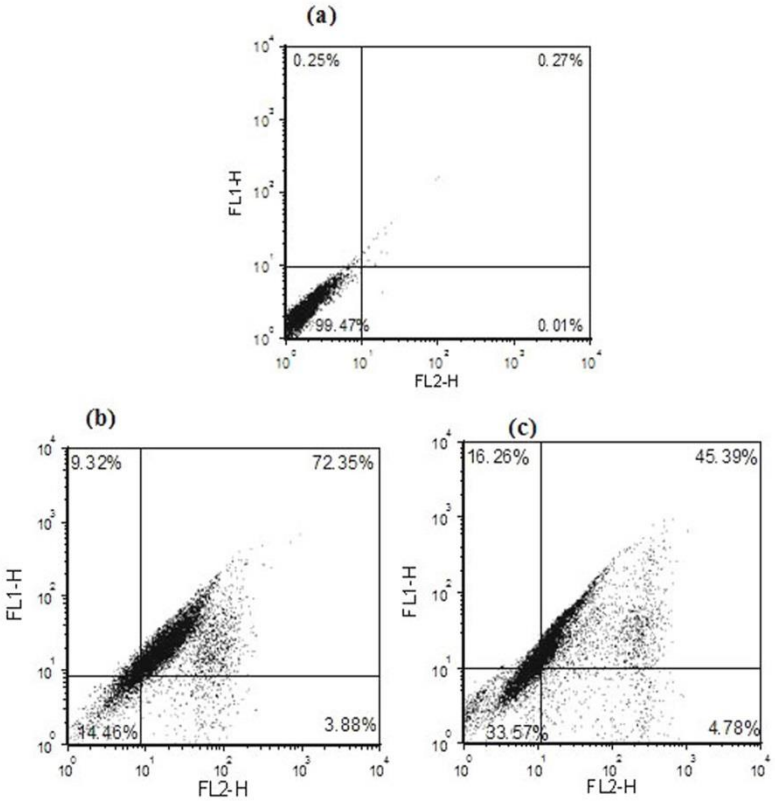

Figure 7: Flow cytometry assay of cell apoptosis in ACHN cells. (a) Control. 24 h after 3-fraction irradiation (b) over 30 min and (c) 45 min

cell survival is seen. The SLDR capacity and speed of a cell are related to the intrinsic radiobiological characteristics of the cell (α/β) and $T_{1/2}$, respectively [23]. ACHN which has low α/β (5.3) and short $T_{1/2}$ (19 min) has high ability to undergo SLDR with increase in the fraction delivery time. In agreement with our finding, Zheng et al. [17, 23] in two separate studies, demonstrated increase in the survival fractions of the nasopharyngeal carcinoma cell lines CNE1 and CNE2 and the human hepatocellular carcinoma cell line HepG2 with prolonged FDTs at a conventional dose/fraction. The authors suggested that SLDR was the predominant factor that decreased cell killing, especially for the cell lines with low α/β and short $T_{1/2}$.

Others reported the enhanced tumor antigenicity and vascular ablation could be possible reasons for additional impact of SBRT [28, 29], but in our experiment, we did not check this possibility. However, Brown et al. [30] concluded that the conventional radiobiological concepts were sufficient to explain the clinical results of SBRT.

Regarding the effect of fractionation, no significant differences could be detected among various fractionation groups when the total radiation time was 15 min. This finding may be attributable to the half time of SLDR of this cell line. Since $T_{1/2}$ (19 min) was longer than the treatment time of 15 min, the SLDR in ACHN cells could be only partially completed during the short delivery fraction time, and therefore, the cell survival would not change appreciably.

With increase in the total radiation time to 30 and 45 min, it was found that with the same total dose, 2- and 3-fraction irradiation led to more cell killing than 1-fraction irradiation. Redistribution of the cell cycle plays a key role in the cell response and can lead to reduced cell survival. The total cell cycle time of ACHN cells is 48 hr (unpublished data), and delivering the second and third fractions after 24 hr would possibly redistribute cells in the G2/M radiosensitive phase and decrease cell survival. We investigated the influence of 3-fraction irradiation and FDT on the cell cycle redistribution; it was found that the percentage of cells in the G2/M phase increased in both 30 and 45 min FDT. Since this phase is a radiosensitive phase of cells, there would be a decrease in cell survival. Consistent with our data, Withers et al. [31] reported that use of two or more fractions per day would allow time for redistribution, and the possibility of irradiating cycling cells in sensitive phases would increase. They also showed that a larger increase in radiosensitivity due to cell cycle redistribution may be expected when smaller doses per fraction are used. Following the preceding study, we investigated the role of 30 or 45 min FDT for 3-fraction in radiosensitivity of ACHN cells by apoptosis. Our data indicated that the rate of occurrence of apoptosis was higher in the FDT of 30 min as compared to that in the FDT of 45 min. It means that prolonged FDT resulted in decrease in the percentage of apoptotic cells. This is consisted with Yao et al. [32] report. They demonstrated that autophagy played the main role in the reduction of the apoptotic cells after irradiation with prolonged FDT via the elimination of radiation-induced ROS. We speculate that with longer FDT (45 min), the repair of DNA and cell cycle progression are more possible than apoptosis in comparison to short FDT (30 min).

There are some limitations in the present study. First, because of concerns about the accuracy of measuring the low survival at higher doses, we could not use the high-dose which other studies have administrated. Second, cell culture experiments do not allow us to determine the effect of hypofractionated radiotherapy on the supporting tissue of the tumors in vivo for example on vascular endothelium.

Conclusion

When the total treatment time increased from 15 min to 45 min in each fraction, tumor cell killing reduced. It seems that the main phenomenon that affects the cell response is SLDR. The effect of the hypofractional dose administered over 15 min may be higher than that for 30 and 45 min. The relative surviving fractions significantly decreased with increase in fraction number over 30 to 45 min of FDT.

Redistribution of the cell cycle plays a key role in the cell response to hypofractionaton and can lead to reduced cell survival. Our data demonstrated that FDTs can affect the apoptosis rate of ACHN cells. These results suggest that in-vitro modelling SBRT irradiation of ACHN cells might yield clinically significant differences in tumor response at a given dose if total radiation time be comparable to the repair halftime of sublethal damage.

Acknowledgment

This study was financially supported by grant: CMRC-101 from Vice-Chancellor for Research Affairs of Ahvaz Jundishapur University of Medical Sciences.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Protzel C, Maruschke M, Hakenberg OW. Epidemiology, aetiology, and pathogenesis of renal cell carcinoma. *European Urology Supplements*. 2012;**11**:52-9. doi.org/10.1016/j.eursup.2012.05.002.
2. Robb VA, Karbowniczek M, Klein-Szanto AJ, Henske EP. Activation of the mTOR signaling pathway in renal clear cell carcinoma. *J Urol*. 2007;**177**:346-52. doi.org/10.1016/j.juro.2006.08.076. PubMed PMID: 17162089.
3. Yu H, Lin X, Wang F, Zhang B, Wang W, Shi H, et al. Proliferation inhibition and the underlying molecular mechanisms of microRNA-30d in renal carcinoma cells. *Oncol Lett*. 2014;**7**:799-804. PubMed PMID: 24520297. PubMed PMCID: 3919943.
4. Mena AC, Pulido EG, Guillen-Ponce C. Understanding the molecular-based mechanism of action of the tyrosine kinase inhibitor: sunitinib. *Anticancer Drugs*. 2010;**21**:S3-11. doi.org/10.1097/01.cad.0000361534.44052.c5. PubMed PMID: 20110785.
5. Kochevar J. Blockage of autonomous growth of ACHN cells by anti-renal cell carcinoma monoclonal antibody 5F4. *Cancer Res*. 1990;**50**:2968-72. PubMed PMID: 2334900.
6. Stinauer MA, Kavanagh BD, Schefter TE, Gonzalez R, Flaig T, Lewis K, et al. Stereotactic body radiation therapy for melanoma and renal cell carcinoma: impact of single fraction equivalent dose on local control. *Radiat Oncol*. 2011;**6**:34. doi.org/10.1186/1748-717X-6-34. PubMed PMID: 21477295. PubMed PMCID: 3094365.
7. Lo SS, Fakiris AJ, Chang EL, Mayr NA, Wang JZ, Papiez L, et al. Stereotactic body radiation therapy: a novel treatment modality. *Nat Rev Clin Oncol*. 2010;**7**:44-54. doi.org/10.1038/nrclinonc.2009.188. PubMed PMID: 19997074.
8. Wersall PJ, Blomgren H, Lax I, Kalkner KM, Linder C, Lundell G, et al. Extracranial stereotactic radiotherapy for primary and metastatic renal cell carcinoma. *Radiother Oncol*. 2005;**77**:88-95. doi.org/10.1016/j.radonc.2005.03.022. PubMed PMID: 15972239.
9. Svedman C, Sandstrom P, Pisa P, Blomgren H, Lax I, Kalkner KM, et al. A prospective Phase II trial of using extracranial stereotactic radiotherapy in primary and metastatic renal cell carcinoma. *Acta Oncol*. 2006;**45**:870-5. doi.org/10.1080/02841860600954875. PubMed PMID: 16982552.
10. Svedman C, Karlsson K, Rutkowska E, Sandstrom P, Blomgren H, Lax I, et al. Stereotactic body radiotherapy of primary and metastatic renal lesions for patients with only one functioning kidney. *Acta Oncol*. 2008;**47**:1578-83. doi.org/10.1080/02841860802123196. PubMed PMID: 18607859.
11. Teh BS, Ishiyama H, Mathews T, Xu B, Butler EB, Mayr NA, et al. Stereotactic body radiation therapy (SBRT) for genitourinary malignancies. *Discov Med*. 2010;**10**:255-62. PubMed PMID: 20875347.
12. Wang X, Xiong XP, Lu J, Zhu GP, He SQ, Hu CS, et al. The in vivo study on the radiobiologic effect of prolonged delivery time to tumor control in C57BL mice implanted with Lewis lung cancer. *Radiat Oncol*. 2011;**6**:4. doi.org/10.1186/1748-717X-6-4. PubMed PMID: 21226899. PubMed

PMCID: 3024935.

13. Benedict SH, Lin PS, Zwicker RD, Huang DT, Schmidt-Ullrich RK. The biological effectiveness of intermittent irradiation as a function of overall treatment time: development of correction factors for linac-based stereotactic radiotherapy. *Int J Radiat Oncol Biol Phys*. 1997;**37**:765-9. doi.org/10.1016/S0360-3016(97)00023-0. PubMed PMID: 9128949.
14. Elkind MM. The initial part of the survival curve: does it predict the outcome of fractionated radiotherapy? *Radiat Res*. 1988;**114**:425-36. doi.org/10.2307/3577116. PubMed PMID: 3287428.
15. Elkind MM, Sutton H. X-ray damage and recovery in mammalian cells in culture. *Nature*. 1959;**184**:1293-5. doi.org/10.1038/1841293a0. PubMed PMID: 13819951.
16. Fowler JF, Welsh JS, Howard SP. Loss of biological effect in prolonged fraction delivery. *Int J Radiat Oncol Biol Phys*. 2004;**59**:242-9. doi.org/10.1016/j.ijrobp.2004.01.004. PubMed PMID: 15093921.
17. Zheng XK, Chen LH, Wang WJ, Ye F, Liu JB, Li QS, et al. Impact of prolonged fraction delivery times simulating IMRT on cultured nasopharyngeal carcinoma cell killing. *Int J Radiat Oncol Biol Phys*. 2010;**78**:1541-7. doi.org/10.1016/j.ijrobp.2010.07.005. PubMed PMID: 21092834.
18. Wang JZ, Li XA, D'Souza WD, Stewart RD. Impact of prolonged fraction delivery times on tumor control: a note of caution for intensity-modulated radiation therapy (IMRT). *Int J Radiat Oncol Biol Phys*. 2003;**57**:543-52. doi.org/10.1016/S0360-3016(03)00499-1. PubMed PMID: 12957268.
19. Fowler JF. The linear-quadratic formula and progress in fractionated radiotherapy. *Br J Radiol*. 1989;**62**:679-94. doi.org/10.1259/0007-1285-62-740-679. PubMed PMID: 2670032.
20. Sterzing F, Munter MW, Schafer M, Haering P, Rhein B, Thilmann C, et al. Radiobiological investigation of dose-rate effects in intensity-modulated radiation therapy. *Strahlenther Onkol*. 2005;**181**:42-8. doi.org/10.1007/s00066-005-1290-1. PubMed PMID: 15660192.
21. Lin PS, Wu A. Not all 2 Gray radiation prescriptions are equivalent: Cytotoxic effect depends on delivery sequences of partial fractionated doses. *Int J Radiat Oncol Biol Phys*. 2005;**63**:536-44. doi.org/10.1016/j.ijrobp.2005.06.010. PubMed PMID: 16168846.
22. Mu X, Lofroth PO, Karlsson M, Zackrisson B. The effect of fraction time in intensity modulated radiotherapy: theoretical and experimental evaluation of an optimisation problem. *Radiother Oncol*. 2003;**68**:181-7. doi.org/10.1016/S0167-8140(03)00165-8. PubMed PMID: 12972314.
23. Zheng XK, Chen LH, Yan X, Wang HM. Impact of prolonged fraction dose-delivery time modeling intensity-modulated radiation therapy on hepatocellular carcinoma cell killing. *World J Gastroenterol*. 2005;**11**:1452-6. doi.org/10.3748/wjg.v11.i10.1452. PubMed PMID: 15770720. PubMed PMCID: 4305686.
24. Kothari G, Foroudi F, Gill S, Corcoran NM, Siva S. Outcomes of stereotactic radiotherapy for cranial and extracranial metastatic renal cell carcinoma: a systematic review. *Acta Oncol*. 2015;**54**:148-57. doi.org/10.3109/0284186X.2014.939298. PubMed PMID: 25140860.
25. Dale RG. The application of the linear-quadratic dose-effect equation to fractionated and protracted radiotherapy. *Br J Radiol*. 1985;**58**:515-28. doi.org/10.1259/0007-1285-58-690-515. PubMed PMID: 4063711.
26. Dale RG. Radiobiological assessment of permanent implants using tumour repopulation factors in the linear-quadratic model. *Br J Radiol*. 1989;**62**:241-4. doi.org/10.1259/0007-1285-62-735-241. PubMed PMID: 2702381.
27. Ning S, Trisler K, Wessels BW, Knox SJ. Radiobiologic studies of radioimmunotherapy and external beam radiotherapy in vitro and in vivo in human renal cell carcinoma xenografts. *Cancer*. 1997;**80**:2519-28. doi.org/10.1002/(SICI)1097-0142(19971215)80:12+<2519::AID-CNCR26>3.0.CO;2-E. PubMed PMID: 9406705.
28. Park HJ, Griffin RJ, Hui S, Levitt SH, Song CW. Radiation-induced vascular damage in tumors: implications of vascular damage in ablative hypofractionated radiotherapy (SBRT and SRS). *Radiat Res*. 2012;**177**:311-27. doi.org/10.1667/RR2773.1. PubMed PMID: 22229487.
29. Dewan MZ, Galloway AE, Kawashima N, Dewyngaert JK, Babb JS, Formenti SC, et al. Fractionated but not single-dose radiotherapy induces an immune-mediated abscopal effect when combined with anti-CTLA-4 antibody. *Clin Cancer Res*. 2009;**15**:5379-88. doi.org/10.1158/1078-0432.CCR-09-0265. PubMed PMID: 19706802. PubMed PMCID: 2746048.
30. Brown JM, Carlson DJ, Brenner DJ. The tumor radiobiology of SRS and SBRT: are more than the 5 Rs involved? *Int J Radiat Oncol*

Biol Phys. 2014;**88**:254-62. doi.org/10.1016/j.ijrobp.2013.07.022. PubMed PMID: 24411596. PubMed PMCID: 3893711.

31. Withers HR. Cell cycle redistribution as a factor in multifraction irradiation. *Radiology*. 1975;**114**:199-202. doi.org/10.1148/114.1.199. PubMed PMID: 1208860.

32. Yao Q, Zheng R, Xie G, Liao G, Du S, Ren C, et al. Late-responding normal tissue cells benefit from high-precision radiotherapy with prolonged fraction delivery times via enhanced autophagy. *Sci Rep*. 2015;**5**:9119. doi.org/10.1038/srep09119. PubMed PMID: 25766900. PubMed PMCID: 4357857.