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Demonstration of bystander response in high dose technique of grid using theoretical calculation by linear quadratic model along with experimental investigations

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► ***Original article***

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ABSTRACT

Background: The Linear Quadratic (LQ) equation as the most common formula in radiotherapy has a debatable accuracy in modeling high-dose effects. The purpose of this study was to demonstrate bystander response of the Grid treatment in SCC cell line, based on both theoretical calculations and experimental investigations. ***Materials and methods:*** The linear quadratic model was used to calculate the equivalent uniform dose (EUD) of a Grid-field with the 10 Gy maximum doses. According to the EUD definition, the identical tumor survival fraction (SF) was expected to obtain from both Grid and open-field single fraction. After observing the difference, the clonogenic and apoptosis assays were exerted to investigate bystander response via medium transfer strategy which was performed from 10Gy-irradiated donors to 1.5Gy-irradiated recipients. ***Results:*** The EUD was equal to 4 Gy and the SF of 4 Gy EUD and 10 Gy Grid-field were 0.1 ± 0.02 and 0.051 ± 0.008, respectively. These findings contradicted the theoretical expectations of their survivals equality. Moreover, the bystander clonogenic cells death enhanced approximately by 2.91 times (statistically significant); highlighting the bystander response role. The apoptotic findings illustrated that the bystander cells experienced an approximately 10% increase and the apoptotic rate confirmed the clonogenic survival result which was less in the EUD than the Grid-field. ***Conclusion:*** Since the SF of the Grid-field was less than the EUD, it revealed the Grid therapeutic advantages plus bystander response manifestation; that was ignored in the LQ equation and may not be demonstrated by sheer theoretical calculations of the modulated-field.

Keywords: *Linear quadratic, grid, bystander, clonogenic survival, apoptosis.*

INTRODUCTION

The most common quantitative formula of dose fractionation relationship in radiotherapy has been Linear Quadratic (LQ) model [(1)]. The LQ model which comprised beneficial properties for radio therapeutic isoeffect-dose predictions could be considered as a suitable method clinically [(2)]. However, despite its frequent application in modeling the effects of radiotherapy at low and medium doses, its accuracy in high-dose levels might be debatable [(3)]. Moreover, the classical LQ model of Lea and Catcheside [(4)] mathematically illustrated the clonogenic survival of cells exposed to a uniform radiation fields.

One radiotherapy technique in high-dose area which should be evaluated from the LQ model validity point of view could be Grid therapy. Wealth of reports indicated that the treatment of bulky and advance tumors might be challenging and traditional radiotherapy fulfilled the aim of maximum tumor control using uniformly entire target volume irradiation. Large-sized tumors treatment considered as a controversial issue due to various biological and technical reasons [5]. The leading cause of concern while implementing open field conventional radiotherapy for bulky tumors might be normal tissue tolerance, so the approach of modulated beam could be an appropriate method in this regard. Spatially fractionated Grid radiation therapy (SFGRT) as an effective curative and palliative hypo-fraction technique could be performed as several narrow beam fields, delivering high dose single fraction of 10-20 Gy in which specific regions of target directly irradiated while neighboring areas locating in valley region shielded from directly high-dose irradiation. Modulated beam of Grid could be established by perforated lead or cerrobend block or multileaf collimator (MLC) system and also hybrid collimation [6-9]. Recently, the advantage of partial volume irradiation as effective as open-field in local and distant area was ascertained in three-dimensional technique of Grid radiation namely 3D LRT [10].

The approach of modulated beam in a single fraction size greater than the standard terminated in normal tissue toxicity reduction plus an appropriate tumor control, so radiobiological elucidation of the Grid-field comparing to the open-field irradiation might be of paramount importance. High-dose hypo fractionation radiotherapy particularly aimed at distinct 4R from the traditional concept [11]. Suggested radiobiological mechanisms occurred in the SFGRT included bystander response, vascular changes, and immunomodulation properties [9]. The non-target phenomenon of the radiation-induced bystander effect (RIBE) considered as a main approach which originally observed by Nagasawa and Little [12]. RIBE has challenged the classical dogma that biological effects occurred in directly irradiated cells, emphasizing on manifestation of radiation-triggered damage in non-directly irradiated cell (bystander cells) via cell signaling of irradiated ones (target cells). The underpinning of the RIBE and its underlying mechanism have been still ambiguous but based on a large body of scientific evidence two mechanistic approaches of signal transmission were included; “medium transfer” and “cell-to-cell contact”.

Although some experimental studies have acquired evidence of survival distinctions between modulated fields and open-field LQ predictions [13-17], it was necessary to examine the LQ equation of high-dose levels in nonuniform dose distribution of Grid treatment. Additionally, theoretical studies have provided evidence of Grid clinical response solely in terms of therapeutic ratios [18]. Therefore, this study was performed to demonstrate bystander response of Grid treatment in human carcinoma cell line of HN5, based on both theoretical calculations and experimental investigations. To investigate bystander response as an ignored part of LQ relationship, the medium transfer strategy was exerted and the clonogenic cell deaths along with apoptosis inductions were studied consequently.

MATERIALS AND METHODS

Theoretical calculations

The standard LQ model considered as a useful tool to predict the clonogenic survival (SF) after a radiation dose (D) (equation 1). The parameters of α and β defined as linear and quadratic terms which are cell-specific parameters and obtained from dose response experiments:

$$SF = e^{-(\alpha D + \beta D^2)} \quad (1)$$

To assess the Grid block dose response theoretically, the standard LQ model could be altered. Gafchromic EBT3 has been applied to perform film dosimetry for dose profiles within Grid-field irradiation (d_{max}: 5 cm and SSD: 100cm, field size: 10cm ×10cm) in Solid Water™

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phantom and isodose curve of the Grid-field was provided (figure 1). The basic assumption of the cells volume under each Grid apertures was that they considered as portions of circular rings shape with the thickness of 0.1 mm. According to the LQ model, the survival fraction (SF) of cells irradiated by Grid-field could be calculated by equation 2:

$$SF_{Tumor}(\text{Grid-field})=\sum A_i\, e^{-(\alpha_{Tumor}\, D_i+\beta_{Tumor}\, D_i^2)} \quad (2)$$

Where the A_i indicates the ratio of the area in the Grid-field, receiving X-ray dose ranging from *Di* and *Di+1* (according to the obtained isodose curve). Based on the assumption of uniform distribution of the cells within the irradiation area, the A_i considered as the ratio of each ring area to the Grid aperture total area (equation 3).

$$A_i = \pi\,(R_{i+1}^2 - R_i^2) \,/\, \pi\, R^2_{max} \quad (3)$$

Where R_{max} is the largest circle radius under one aperture and is half of the center-to-center distances between the Grid apertures.

Equivalent uniform dose (EUD) in Grid therapy was considered as the absorbed dose from an open-field single fraction that resulted in the similar tumor survival fraction (SF) to the Grid-field (equations 4 and 5).

$$SF_{Tumor}(EUD) = e^{-(\alpha_{Tumor}\, EUD + \beta_{Tumor}\, EUD^2)} \quad (4)$$

And therefore;

$$\sum A_i\, e^{-(\alpha_{Tumor} D_i+\beta_{Tumor} D_i^2)}=e^{-(\alpha_{Tumor}\, EUD+\beta_{Tumor}\, EUD^2)} \quad (5)$$

To calculate EUD, it was crucial to extract α and β constants from the Survival Fraction (SF) curve (equation 6). After the EUD calculation, the tumor cell line could be irradiated by the open-field EUD.

$$\alpha_{Tumor} EUD+\beta_{Tumor} EUD^2+\ln(SF_{Tumor}(\text{Grid-field}))=0 \quad (6)$$

Experimental measurements

Human Cell Culture

Since the potential role of Grid treatment in nasopharyngeal carcinoma (NPC) as an endemic case in many low-to-middle-income countries has been considered [19], human head and neck squamous cell carcinoma (HN5) cell line was selected for this research as a challenging and radio-resistant tumor cell line which was obtained from the National Cell Bank of the Pasteur Institute (Tehran, Iran). HN5 as an adherent cell line was seeded in T-25 culture flasks to grow exponentially. The culture flasks consisted of 5 ml culture medium containing Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Invitrogen, UK) supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen, UK) and 1% penicillin-streptomycin (Gibco, Invitrogen, UK), maintained in humidified incubator providing 5% CO^2 at 37°C.

Grid-field Irradiation

48h before Grid irradiation, 5×10^5 cells were seeded in triplicate cell culture dishes with 9cm in diameter and 2 cm height to simulate size of bulky tumor. The Grid block used in this experiment was constructed based on the previous survey [20] and then used clinically at Cancer Institute of Imam Khomeini medical center (Tehran). The lead-made Grid block properties included the thickness of 7.5cm, 145 circular fields, 13 mm in diameter with 17 mm center-to-center distance. Given the experimental setup validations, dosimetry measurements were previously carried out by use of EBT3 Gafchromic film [20]. The Grid block mounted on the Varian 2100C linear accelerator (Linac) for a 6 MV photon beam (figure 2). Cell dishes were located between 1.6 cm (top) and 6 cm (below) PTW water equivalent slab phantoms and irradiated with the clinical dose of 10 Gy, SSD (Source to Skin Distance) of 100 cm, field size of 20 cm × 20 cm at the isocenter. Tissue-equivalent bolus was used surrounding the dishes, considering full scatter conditions.

Medium transfer Irradiation

Exponentially growing cells were seeded in T-25 culture flasks containing 5 ml culture medium 48h before treatment in triplicate manner. Regarding medium transfer as a gold standard of bystander response evaluation, it was vital to expose the cells based on the clinical Grid peak-to-valley dose profile curve illustrated

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in Figure 3. The clinical Grid dose ranged from 10 to 20 Gy which 10 Gy Grid irradiation was selected in this experiment. According to the dosimetric confirmations, aperture center (peak region of dose-profile curve) received 10 Gy at D_{max}; while shielded center (valley region of dose-profile curve) received an average dose of 1.5 Gy (due to center-to-center distance variations). PTW water equivalent slab phantoms with total thickness of 2 cm at the top and 6 cm under the cell dishes were placed to achieve SSD (Source to Skin Distance) of 100 cm. Consequently, donor and recipient cells were irradiated by 10 Gy and 1.5 Gy respectively using the Varian 2100C linear accelerator (Linac) of 6 MV photon beam, field size of 20 cm × 20 cm at the isocenter. The Linac has been calibrated according to the IAEA TRS 398 dosimetry protocol.

Bystander response evaluation based on medium transfer

To achieve maximum bystander signal, the cells were incubated for 4h and then the medium of recipients were discarded and replaced by conditioned medium (CM) which described as a filtered medium of donors by aid of 0.22μm filter. Therefore, all cells and cellular fragments were removed from the medium and just soluble factors of it remained.

Clonogenic survival assay

By passing 24 h from the Grid irradiation or the medium transfer, HN5 cells in sparsely definite numbers of 100, 200 and 500 were seeded in six-well plates and incubated 12 days. To obtain the survival curve, megavoltage (6 MV) X-irradiation was implemented with doses of 0 (control), 1, 2, 4, and 6 Gy according to the setup of medium transfer irradiation. Cells were washed with PBS, trypsinized, counted using "Trypan Blue" dye (Sigma- Aldrich, USA), and then seeded in Petri dishes in appropriate densities proportional to the radiation doses. Then the cells kept in the humidified 37 °C incubator for 12 days. After colonies (>50 cells) formation, they were stained with 0.5% crystal violet (Sigma- Aldrich, USA) and counted using Inverted phase microscopy (CETI, Belgium). Plating efficiency percentage (%PE) defined as the ratio of the number of counted colonies to the seeded cells multiplying by 100. Survival Fraction (SF) also calculated by normalizing efficiencies of irradiated groups to the unirradiated control ones. Survival curve plotted using OriginLab Software (Version 2018) which the log SF considered vs. the radiation dose and fitted to the linear-quadratic model (LQ model) according to equation 1 that SF is the cell survival fraction as a function of D (radiation dose), α and β constants correspond to the linear and the quadratic parts of the curve. The parameters including α, β, SF2, D10, D20, D37, D50, D80, and D90 were extracted from the curves. SF2 defined as the survival fraction at 2 Gy whereas D10, D20, D37, D50, D80, and D90 described the doses related to the survival fractions of 10%, 20%, 37%, 50%, 80% and 90% respectively.

Annexin V-FITC apoptosis staining assay

To examine whether the apoptotic induction as another cell death alternative followed the same trend of clonogenic cell survival, Annexin staining assay was conducted. 4 h after the irradiation medium transfer was performed and at the specific time of 24 h as an apoptosis induction appropriate interval [21], Annexin apoptotic assay was initiated by the use of FITC Annexin V Staining Kit (BioLegend), according to the manufacturer's instruction. Samples were analyzed for the apoptotic cells presence by the use of BD FACS Calibur flow cytometer (BD Bioscience). 10,000 calls per each sample were evaluated and the resultant data were analyzed using the BD Cell Quest Pro software.

Statistical analysis

All data were expressed in terms of mean values ± SEM (standard error of the mean) and analyzed statistically by the one-way analysis of variance (ANOVA) test. The P-values of less than 0.001 (***), 0.01 (**), and 0.05 (*) were considered as a significant level. Statistical analysis was performed using SPSS software (version 24).

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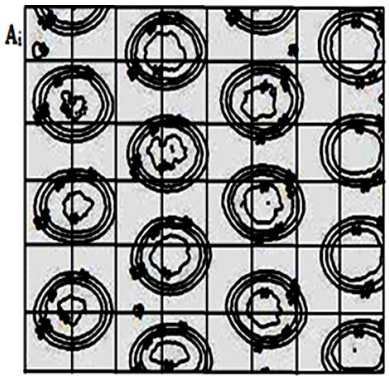

Figure 1. Film isodose curve of the Grid -field in which Ai represented the pixel area; for better illustration it was displayed in larger size than the basic volume assumption.

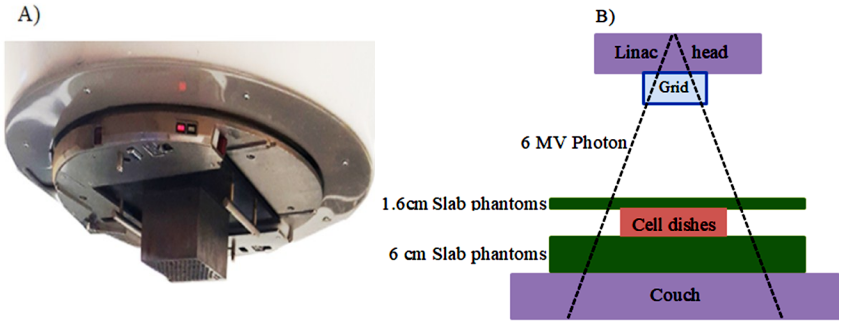

Figure 2. A) The clinical Grid block mounted on the Varian 2100C linear accelerator (Linac); **B)** Schematic view of the irradiation setup.

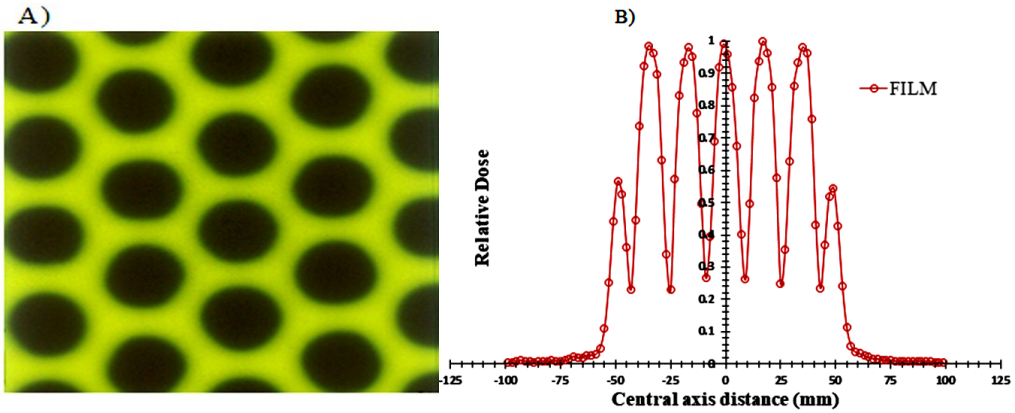

Figure 3. Gafchromic EBT3 film exposed by the modulated field of Grid irradiation. Darkened and lightened regions represented the aperture and block areas of the irradiated field. **B)** The peak-to-valley dose profile curve under Grid holes which was measured using EBT3 Gafchromic film. The medium transfer irradiation was performed based on the proposed curve.

RESULTS

Theoretical EUD calculations

The resultant α and β parameters as well as the survival fraction of 10 Gy Grid-field were placed according to equation 6. α and β constants were extracted from HN5 survival curve that fitted to LQ model (table 1). The SF of Grid- field was obtained based on the figure 4 .Consequently, the calculated EUD according to equation 5 was equal to 4 Gy which indicated that the HN5 survival fraction resulted from single fraction dose of 4 Gy should equal to those from 10 Gy Grid-field. The SF of 4 Gy open-field was 0.1 ± 0.02 (figure 4) while the SF of 10 Gy Grid-field was 0.051 ± 0.008.Therefore, the survival fraction of 10 Gy Grid-field was not the same as the single fraction of open-field (EUD:

SF_{HN5} (10 Gy Grid-field) < SF_{HN5} (4Gy EUD)

The therapeutic ratio of the Grid-field irradiation could be defined as the ratio of the tumor cell survival fraction under an open field with equivalent dose of EUD to the survival fraction under Grid-field irradia-tion (equation 7).

$$TR = SF_{Tumor}(EUD) / SF_{Tumor}(Grid\text{-}field) \quad (7)$$

The proposed ratio was equal to 1.96 which emphasized on the Grid-field enhancement cell death effect. According to obtained results, it

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could be concluded that there might be the radiobiological effects which were ignored in the LQ model. Since the probable response of bystander might be indicated in hypofractionation radiation technique of the Grid treatment, we evaluated the RIBE in the medium transfer strategy.

Clonogenic survival assay

The calculated SF2 as the standard method of radiosensitivity prediction was 0.5±0.03, highlighting the radioresistance of HN5. The survival curve fitted to the LQ model illustrated in figure 4 and different extracted parameters were indicated in table 1. According to the table 1, more value of the quadratic parameter (ß) than linear one (α) confirmed that HN5 could be considered as the radioresistant cell line as well. Obvious reduction trend of clonogenic survival by increasing X-ray dose observed as expected. The SF of 1.5 Gy medium transfer decreased approximately by 2.91 times comparing to 1.5 Gy open-field. Therefore, the results highlighted the probable role of the RIBE as defined by statistically significant survival decrease ($P<0.05$) in bystander groups comparing to directly-irradiated cells. Moreover, the SF reduction resulted from the 1.5 Gy media transfer bystander was more than those obtained from 2 Gy open-field, implying the cell death enhancement ratio resulted from RIBE more pronounced than sheer 2Gy irradiation. As illustrated in the figure 4, the SF of 10 Gy Grid-field was less than 4 Gy open-field (EUD) which leaded us to the fact that theoretical calculations based on the LQ model should be altered; due to the biological effects including bystander response and etc. that were ignored in the LQ equation. Noteworthy, there was no statistically significant difference between the SF of 10Gy open-field and Grid-field, implying another confirming evidence of the Grid therapeutic advantage.

Annexin staining assay

The apoptotic results indicated the overall ascending trend of apoptosis induction parallel with increasing dose. Moreover, the slight increasing trend was illustrated in the adjacent cells that experienced medium exchange compared to sheer the 1.5 Gy open field, which was not statistically significant (figure 5). HN5 bystander cells had approximately 10% apoptotic enhancement than 1.5 Gy directly-irradiation cells. Noteworthy, the apoptotic rate of EUD (4 Gy open-field) was less than 10 Gy Grid-field which was consistent with the clonogenic cell survival results.

Table 1. Different parameters extracted from survival curves fitted to the LQ model.

	α (Gy^{-1})	ß (Gy^{-2})	D90 (Gy)	D80 (Gy)	D50 (Gy)	D37 (Gy)	D20 (Gy)	D10 (Gy)	SF2 (Gy)
HN5	0.089	0.121	0.611	1.036	2.051	2.52	3.285	4.006	0.5

α and β defined as linear and quadratic parameters of LQ model. D90, D80, D50, D37, D20, D10: radiation doses related to the survival fractions of 90%,80%,50%,37%,20% and 10%; SF2: Survival Fraction at 2 Gy.

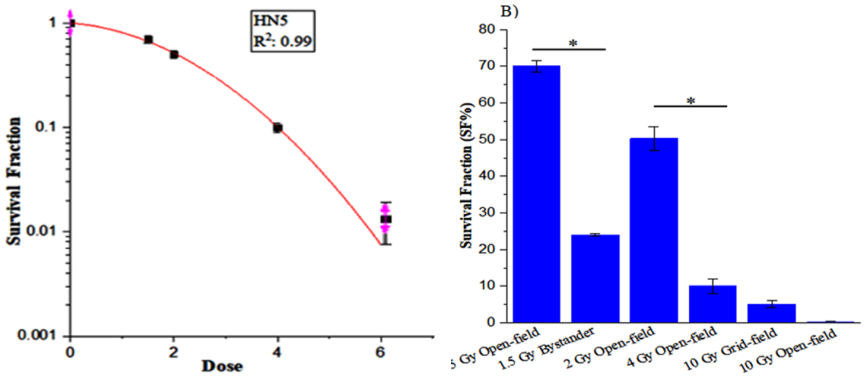

Figure 4. A) Survival curve of HN5 cell line irradiated with graded dose of X-ray. **B)** Clonogenic cell survival in different HN5 groups. Open-field: cells irradiated by uniform dose distribution; 1.5 Gy bystander: cells received uniform dose distribution of 1.5 Gy and experiencing medium transfer from those irradiated by 10 Gy open-field; 2 Gy Open-field defined as SF2; 4 Gy Open-field considered as EUD; 10 Gy Grid-field: total cells under aperture and block regions of Grid irradiated by nonuniform dose distribution of 10 Gy; *: $P < 0.05$.

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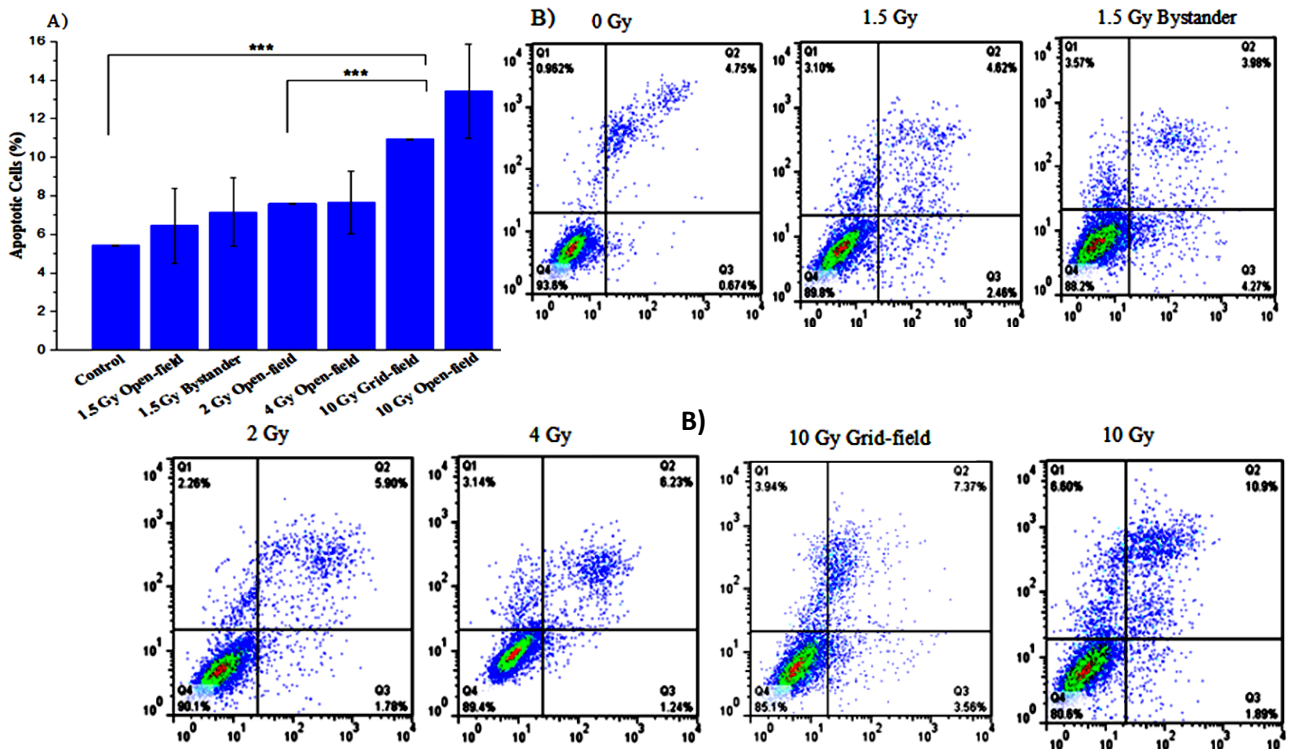

Figure 5.A) The apoptotic cells at 24 h after irradiation, stained with Annexin V–FITC and PI and **B)** Scatter plots of apoptosis in different groups of HN5. (Data expressed as mean ± SEM of three independent experiments).

DISCUSSION

In this study, the linear quadratic model was used to calculate the equivalent uniform dose (EUD) of a Grid-field with the maximum dose of 10 Gy. According to the EUD definition, the identical tumor survival fraction (SF) was expected to obtain from both Grid and open-field single fraction. After observing the difference between the theoretical calculations and experimental results, the bystander response as the most probable ignored term of the LQ relationship for a Grid modulated field was evaluated via medium transfer strategy. To examine whether the apoptotic induction followed the same trend of clonogenic cell death or not, Annexin V-FITC apoptosis staining assay was performed as well. Obtained results indicated that theoretical measurements predicted the equality in 10 Gy Grid-field and 4 Gy open-field (EUD) survivals according to the α and β parameters of the LQ model extracted from the HN5 survival fraction. Conversely, based on the experimental examination the clonogenic survival of 10 Gy Grid-field was less than 4 Gy open-field, implying that LQ predictions was not concisely true in high-dose modulated beam of Grid. Therefore, the bystander effect as a most probable cause of cell death enhancement in the Grid-field compared to the EUD was evaluated based on the medium transfer strategy. Since in SFGRT, the bystander cells are defined as the tumor cells located in the valley regions of the Grid dose profile, consequently the cell survival of Grid-field treatment could imply the cell death enhancement via bystander response comparing to the EUD or open-field irradiation. The results emphasized on the RIBE occurrence in a way that clonogenic cell death enhanced in 1.5 Gy bystander HN5 cells approximately 2.91 times than sheer 1.5 Gy open-field ones.

To date, overwhelming evidence has been focused on different aspects of the Grid design and dosimetric validations [20, 22-25] along with clinical outcomes including acceptable tumor local control and pathological responses [8, 26, 27]. The outstanding merit of the SFGRT could be

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regarded as high-dose delivery without exceeding the tolerance dose of organ at risks [9]. From the radiation biology point of view, the RIBE elucidation after the SFGRT could be considered as a huge help to justify satisfying response of the modulated Grid treatment parallel with the open uniform field. The RIBE observation in the current study has been in line with Asur et al investigation of cell death decreasing rate in bystander group after 10 Gy Grid irradiation [28]. Clinical data of Grid therapy expressed the promising local control in Head & Neck SCC, whereas in contrast the conventional fractionation regimen has not achieved the identical success [29] and the importance of the Grid treatment in Head and neck SCC was previously ascertained [19]. Since our selected cell line of HN5 had SF2 value in radioresistant range (SF2 > 0.4) and several resultant parameters particularly α and β emphasized on its radioresistance nature, consequently in agreement with some reports [16, 18] we observed bystander enhancement role in Grid treatment of radioresistant tumor cell line of SCC. Additionally, the therapeutic advantage of the Grid-field irradiation could be defined as an increase in ratio of the tumor cell survival fraction under an open field with the equivalent dose of EUD to the survival fraction under Grid-field irradia-tion. Based on the obtained ratio, we observed approximately 2 times increase in the Grid-field clonogenic cell death comparing to the EUD. Moreover, there was no statistically significant difference between 10 Gy Grid-field and open-field. Consequently, the obtained results of theoretical calculation combined with experimental investigation of the current work highlighted the Grid-field therapeutic advantage which was even comparable to high-dose open-field irradiation.

The chief cause of prevalent use of the LQ equation could be its biological response basis in tumor control and normal tissue complications attributed to cell death [2]. LQ as a plausible model for dose-per-fraction range from 2 to 10 Gy might be less accurate and reliable above 10 Gy [2]. The LQ model has been defined as mechanistic approach; based on the fact that the cell death consequent was formed because of misrepair of DNA damages particularly DNA double strand breaks (DSBs) [2]. However, it has been discussed that the α and β constants of LQ model did not indicate the precise radiobiological elucidation of underlying mechanisms [30]. The in-vitro investigation of irradiated CHO cells has stated that the use of DNA flowcytometry for counting cell numbers, rather than colonies resulted in more precise survival curve with well-fitted data to the LQ model in dose range of 2 to 7 Gy [31]. One *in-vitro* study reported that the quality of colony assay-based data fitting to the LQ model did not show the significant decrease until doses more than 15 Gy [32]. Taking these issues into considerations and regarding the fact that DSBs could be terminated in the mitotic cell death and/or apoptosis, we also investigated apoptotic formation to assess its trend particularly in the Grid-field and EUD irradiation plus the medium transfer study. As a result, apoptotic inductions had a slight decreasing trend by increasing dose particularly in the medium transfer evaluation compared to the clonogenic survival. However, the apoptotic rate of EUD (4 Gy open-field) was less than 10 Gy Grid-field which confirmed the clonogenic cell survival results as well.

Several studies concluded the altered model considering the bystander response in the modulated radiation field. To achieve the mathematical predictive model particularly considering the RIBE in Grid treatment, an *in-vitro* investigation using high definition multileaf collimators (HDMLCs) to generate Grid pattern was performed and the extended linear quadratic (LQ) model was finally developed [17]. Additionally, the mathematical study was performed; comprising two sets of α; β parameters to separate the bystander response from the direct effects of radiation. The results ascertained that the bystander component in cell death was significant which should not be ignored [33]. A computational Monte Carlo model of cellular response to the modulated field could incorporate the damage from both direct radiation and intercellular communication of the bystander signaling as well [34]. Peng *et al.* established three different bystander response models in gradient radiation fields and their

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models gave better fitting to the observed cancer cell survivals in uniform and modulated fields than the classical LQ model [35]. Based on the current investigation, because α and β parameters of the LQ model were extracted from uniform open-field, as a result, it could be predicted that the LQ equation couldn't be a precise formalism for the modulated field of Grid and therefore may not demonstrate the non -target response of bystander. However, various radiobiological explanations might be involved in the SFGRT that bystander response was considered as the major one. The need for the predictive models design incorporating bystander effects for cancer treatments improvement might be of paramount importance. Consequently, establishing an accurate model comprising appropriate terms for radiobiological phenomenon to predict better response of an advanced radioresistance tumor to high-dose modulated Grid beam waited more research in the future.

CONCLUSION

As a conclusion, the combination of theoretical measurements and experimental evaluations of the current work provided evidence that the survival fraction of Grid-field was less than EUD and revealed the Grid therapeutic advantages plus manifestation of the bystander response that was ignored in the LQ equation; which may not be demonstrated by sheer theoretical calculations particularly of the modulated field. These findings leaded us to the fact that the predictive calculations based on the LQ model for the nonuniform dose distribution of Grid should be altered, incorporating more precise parameters of the biological effects including bystander response. However, clarifying these parameters required further investigations and confirmations.

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