# Effect of dose rate on antitumor activity in hypoxic cells by using Flattening Filter Free beams

# (Flattening Filter Free における線量率と抗腫瘍効果の相関関係に関する評価と解析)

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*Contents*

# Chapter 1
# Introduction

## 1.1 The concept of radiation therapy

Radiation therapy is one of the treatment method selected in cancer treatment along with surgery and chemotherapy. One in two of all cancer patients receive radiation therapy during the treatment course (1). The concept of radiation therapy is to radiate the tumor with appropriate prescribed dose and to spare risk organ from unnecessary radiation at the same time. It is well known that the radiation therapy usually does not carry prescribed dose at single fraction. Dose prescribed by physician is usually given by the patient in multiple fractions to treat cancer. The reason for fractionated irradiation is applying the difference of cell sensitivities between tumor and risk organ around the tumor (2, 3) (Figure 1).

## 1.2 Stereotactic Body Radiation Therapy

Recently, there is a technique to treat the cancers affecting the thoracic region in few fractions, usually less than five fractions, with large

dose per fraction. This is known as Stereotactic Body Radiation Therapy (SBRT). As mentioned above, SBRT uses large dose per treatment fraction, it results in an increase in treatment time. It causes to limit the determination of patient status. Furthermore, the intrafractional motion during the treatment may also limit the dose reaching the target during the procedure (4). And it may give unnecessary radiation to the organ at risk. SBRT is mainly employed in the treatment of cancers affecting the abdominal or thoracic region where respiratory movement occurs during treatment. To maintain the dose coverage, treatment planner usually adds the margin to the target volume to take care the organ motion during treatment. Recently, there is an advanced technology that is able to account for the respiratory movement in order to minimize normal tissue complications from internal organ displacement (5). It monitors patient respiration cycle and triggers the treatment beam on and off automatically. The technique is known as respiratory gated treatment (6). By using respiratory gated treatment technique, treatment beams automatically turn off if the patient's respiratory motion differentiates from predefined respiratory area. Thus, it causes the longer treatment time, and it needs to

be short enough for the patient comfortable.

## 1.3 Characteristics of flattening filter free beams

There is an advanced technology equipped with radiation treatment machine, which is flattening filter free (FFF), to maximize the dose rate at the maximum of four times higher than normal dose rate (7-11). Flattening filter (FF) exists inside the gantry head after the electron hits the high atomic number of target to generate photon. After the generation of photon, it passes FF to flatten the beam. The technology of FFF removes FF, so the photon does not attenuate by the FF. As the result, FFF can produce high dose rate, thus it can shorten the treatment time to improve patient comfort and total throughput (Figure 2). Previous report concludes that using FFF in SBRT shorten the average treatment time from 46 minutes to 26 minutes (12). Because of physical aspect of FFF, where it removes flattening filter from the gantry head, it produces less outside field dose to the patient from scattering radiation (13). These advantages led the clinicians to implement the use of FFF clinically (14-19). However, there may be a question from radiation biology point of view that the radiating significantly large dose at

the short amount of time may cause different effect on cell survival.

## 1.4 Biological effect of using FFF beams

There is a report that mentions increasing surface dose was observed in treatment using FFF (20). Also there are reports from Mu et al. (21) and Moiseenko et al. (22) that the cell survival rate was higher when the total time spent irradiating the cell was longer. It indicates there is an effect on cell survival by irradiation time. Studies conducted by Lohse et al. (23) reported the effect of high dose per pulse on cancer cell survival comparing 10 MV X-rays with flattening filter (10 X) and FFF (10 X FFF) by using the dose of 5 Gy or 10 Gy. They concluded that using higher dose rate results in the lower the cell survival, especially for irradiation of 10 Gy. At the prescribed dose of 5 Gy, they had not observed any significant difference in cell survival rates when irradiation was carried out using 10 X and 10 X FFF. However, using irradiation of 10 Gy and above, it was seen that the cell survival rate decreased as the prescribed dose increased. These findings are in contrast to recent reports from Karan et al. (24) who showed that there is no significant difference in cell survival rate when the same energy was used with or

without the flattening filter at the same prescribed dose. Therefore, it is still unclear as to whether the usage of high dose rate using FFF beams affects the cell survival rate or not.

## 1.5 Antitumor activity in hypoxic cells using FFF beams

The cause of cell damage by radiation can either be due to direct or indirect action of radiation on DNA. It is known that about two-thirds of the damage inflicted on DNA by low-LET ionization occurs indirectly (25). In indirect action, radiation hits water molecules leading to the production of reactive oxygen species (ROS). Double strand breaks (DSBs) in DNA caused by direct or indirect action of radiation, which trigger cell damage, are also very common. The relationship between DSBs and irradiation dose has been shown to be linear by Nunez et al. (26). It is the general consensus that the solid tumor cells are under hypoxic conditions and are much more resistant to radiation because the availability of oxygen results in DNA damage due to its interaction with hydroxyl radicals (27-28). As mentioned earlier, studies investigating the effect of dose rates employing FFF beams are in limited number. Furthermore, previously reports do not consider the cell oxygenation

status at the time of research. Studies focusing on the use of FFF beams for treatment of hypoxic cancer cells are yet to be conducted. We suggest that the cell oxygenation conditions should be considered in experiments aimed at studying the effect of dose rate, when using FFF beams, due to the hypoxic nature of many tumors. Clearly demonstrating the effect of FFF on hypoxic cancer cells would provide invaluable information to the radiation oncologist. The purpose of the present study is to evaluate the effect of dose rate when using FFF beams on cells with a different oxygenation status, while considering antitumor activity. Additionally, we also carried out APF and γH2AX assays to determine the mechanisms of cell damage.

# Chapter 2
# Materials and Methods

## 2.1 Cell cultures

EMT6 mouse mammary tumor cell line, a widely used model in radiation biology, was obtained from Kyoto University (Kyoto, Japan). The cells were cultured in Eagle's MEM medium (Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 10 % fetal bovine serum (JR Scientific Inc., Woodland, CA, USA). The cells were incubated at 37°C, with 5% $CO_2$ in a subconfluent state. Aliquots were collected from the suspension medium containing $4\times10^4$ cells/mL, and 500 μL of the cell suspension was dispensed into test tubes. This was then treated with 95% $N_2$ and 5% $CO_2$ for 15 min to simulate hypoxic conditions. We confirmed that the tumor cells were under a sufficient hypoxic state by calculating the oxygen enhancement ratio (OER). OER was calculated by dividing the irradiation dose for hypoxic cells by the irradiation dose for aerobic cells required to give a surviving fraction of 10 %. The observed OER under hypoxia after 15 min of exposure to $N_2$ was 3.12.

## 2.2 Irradiation

The TrueBeam linear accelerator (Varian Medical Systems, Palo Alto, CA, USA) was used for irradiation. The cell lines were irradiated to the prescribed doses of 2 Gy and 4 Gy with nominal energy of 10 X without flattening filter (10 X FFF). Different dose rates (6.27 Gy/min, 12.00 Gy/min, and 18.82 Gy/min) were selected to irradiate the absorbed dose. Following irradiation, the test tubes were centrifuged at 1,500 rpm for 10 min to fix the cells. The cells were re-suspended and 500 cells were placed in three dishes for each experiment. All dishes were then incubated at 37°C for 7 days to allow colonies to form. After incubation, cells were washed to remove any leftover media, and fixed on the dishes by adding 2 mL of methanol for 10 min. Following the removal of methanol, 2 mL of 5% Giemsa stain was added and the colonies were stained for 60 min. The number of colonies was counted manually.

## 2.3 Reactive oxygen species detection by APF assay

To support the results from the colony formation assay, an APF assay (Sekisui Medical Co. Ltd., Tokyo, Japan) was performed. A 5 -mM solution of

APF was diluted with 100 mM sodium phosphate buffer to attain a concentration of 100 μM and dispensed into test tubes, each containing 500 μL. After irradiating of 2 Gy at different dose rates (6.27 Gy/min, 12.00 Gy/min, and 18.82 Gy/min), the cells were placed into a multi-well black plate (100 μL per well). Fluorescence was measured using excitation and emission wavelengths of 490 nm and 515 nm, respectively. Non-irradiated cells were prepared as the control group.

2.4 DNA DSBs detection by γH2AX assay

We used the γH2AX assay to support data from the experiments described above. One day prior to treatment, cells from the EMT6 cell line were placed onto a slide chamber at a concentration of $2\times10^4$ cells/mL. Following irradiation, cells were fixed in 4% paraformaldehyde (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) for 10 min. Subsequently, fixed cells were permeabilized with 0.5% Triton-X100 (Sigma-Aldrich Japan, Tokyo, Japan) in phosphate-buffered saline (PBS) then blocked with 1.0% bovine serum albumin (BSA) and 0.2% Triton-X100 in PBS. For the γH2AX colocalization experiments, cells were incubated with anti-phospho-histone H2AX (Ser139), antibody clone JBW301 (Merck KGaA, Darmstadt, Germany),

and donkey anti mouse IgG antibody-fluorescein isothiocyanate (FITC) conjugate (Merck). Cells were then stained with 4′, 6-diamidino-2-phenylindole (DAPI) (Dojindo, Kumamoto, Japan) in PBS for 15 min, and the slides were mounted with *Malinol (Muto Pure Chemicals Co., Ltd.*, Tokyo, Japan). The γH2AX foci were counted manually in the nuclei of 100 cells using a BZ-X700 fluorescence microscope (Keyence, Osaka, Japan) following 2 Gy irradiation; the data shows the average value per cell. We tracked γH2AX foci at 1 h and 24 h of 2 Gy irradiation under hypoxic and aerobic conditions, to consider the possible involvement of DSB repair.

### *Statistical validation*

The data represents the results of assays performed in triplicate. Data are expressed as mean and standard deviation (SD) values. The statistical significance of the differences between the results of the independent experiments was analyzed using the *t*-test of Excel 2010 (Microsoft, Washington, USA). A *p*-value of $<0.05$ was considered statistically significant.

# Chapter 3
# Results

## 3.1 Cell survival

The average cell surviving fractions (SF), plating efficiency (PE), and SD for dose rates 6.27 Gy/min, 12.00 Gy/min, and 18.82 Gy/min are presented in Table 1. The effect of dose rates on cell survival in hypoxic and aerobic cells is plotted in Figure 3(a). Using hypoxic cells, we observed that increasing the dose rate, while using FFF beams, showed an increase in antitumor activity in a dose rate-dependent manner. This suggests that a mechanism exists to induce damage in hypoxic cells involving higher dose rates. Conversely, there were no significant dose rate-dependent differences with respect to cell survival in aerobic cells. The comparison of cell survival between hypoxic and aerobic cells is depicted as a histogram in Figure 3(b). At the prescribed dose of 4 Gy, resistance to radiation was observed in hypoxic cells.

## 3.2 Reactive oxygen species detection by APF assay

To further investigate the cause of cell damage, we performed the APF

assay to detect formation of ROS. The ratio of fluorescence intensity for the control, and dose rates of 6.27 Gy/min, 12.00 Gy/min, and 18.82 Gy/min are 1.78, 1.88, and 1.94 for hypoxic cells, and 2.01, 2.10, and 2.15 for aerobic cells, respectively (Figure 4). The SD value is 0.051, 0.057, and 0.047 for hypoxic cells and 0.029, 0.019, and 0.009 for aerobic cells, respectively.

### 3.3 γH2AX assay

At the prescribed dose of 2 Gy, the number of DNA DSBs at different dose rates and level of cell oxygenation were determined using the γH2AX assay at 1 h and 24 h following irradiation (Figure 5). There are significant differences in the number of DNA DSBs between irradiated and non-irradiated cells (Figure 6). The number of DNA DSBs increased proportionally with the dose rate. By comparing the time course of the number of γH2AX foci under hypoxic condition, there is no significant difference between 1 h and 24 h following irradiation (Figure 6(a).). The SD values for hypoxic cells at zero radiation, and at dose rates of 6.27 Gy/min, 12.00 Gy/min, and 18.82 Gy/min are 0.584, 1.400, 1.546, and 1.728 at 1 h; and 0.486, 1.266, 1.505, and 2.467 at 24 h, respectively. Using aerobic cells, the number of γH2AX foci

significantly decreases after 24 h of irradiation compared to 1 h of irradiation (Figure 6(b)). The SD values for aerobic cells at zero radiation and the different dose rates are 0.852, 1.715, 3.696, and 0.949 at 1 h; 0.125, 0.688, 0.643, and 0.725 at 24 h, respectively.

# Chapter 4
# Discussion and Conclusion

In this study, we evaluated the effect of different dose rates on cell survival in aerobic and hypoxic cells by using FFF beams. The effects of dose rates on different cell types have been previously studied by various groups. We suggest that the cell oxygenation condition needs to be considered in experiments aimed at studying the effect of dose rate when using FFF beams. From Figure 3(a), it is evident that increasing the dose rate results in increased antitumor activity in hypoxic cells. Therefore, a correlation exists between the dose rate and antitumor activity in a dose-dependent fashion. The present study was carried out in accordance with the study to evaluate the effects of dose rate variation on cell survival using a 250-kV irradiator (29). Those results can be explained that the damage occurred over time using lower dose rate compared to the one occurred short time using higher dose rate, may have a much time to repair DNA damage during treatment. On the other hand, those observations were not seen from

the previous report from Karan et al. [24], where they had not seen the dose rate effect on cell survival by using FFF beams. In our study, we did not observe any dose rate effects on cell survival in the cells without hypoxic treatment, which is in agreement with the study reported by Karan et al. [24]. We also compared cell SF between hypoxic and aerobic conditions and it demonstrated that the hypoxic cells were more resistant to radiation than aerobic cells. This observation can be explained based on the fact that hypoxic cells showed less DNA damage or had time to repair the damaged DNA due to the lack of oxygen. These conclusions are supported by the results of Bristow and Hill (30) and Matthews et al. (31). In aerobic cells, the presence of oxygen could aid in the generation of hydroxyl radicals, thereby no neutralization of ROS is observed, and as a result DNA is damaged (32). The OER of this study was 3.12 for treatment with $N_2$ gas for 15 min, which concurs with data by Rockwell and Kallman (33). Our data shows a significant difference between the cell survival of aerobic and hypoxic cells as shown in Figure 3(b). The question remains as to why the SF is related in a dose rate-dependent manner

only under hypoxic conditions; we therefore performed several assays. As explained previously, the mechanism of cell damage occurs mainly due to direct or indirect interaction with ROS. To determine ROS production, we measured fluorescence of APF using the procedure described by Setsukinai et al. (34). The data showed that there was significant difference in fluorescence intensity as a function of dose rate on both hypoxic and aerobic cells. This indicated that ROS production, which could be hydroxyl radical, peroxynitrite or hypochlorite, from the interaction of ionizing radiation with water molecules, increases at higher dose rates. In general, the number of ROS produced by ionizing radiation depends on the dose, not the dose rate. Our data clarifies that the use of high dose rate, for short irradiation time, causes more efficient generation of ROS per unit time compared to using lower dose rates. To test the hypothesis that an increase in ROS production will increase DNA damage, we carried out the γH2AX assay to ascertain whether any DNA DSBs were induced. The data from the γH2AX assay are presented in Figure 6. From the figure, it is clear that there is a significant difference between the

number of DNA DSBs in cells that are irradiated with different dose rates to those that have undergone zero irradiation, with the number of DNA DSBs increasing as a function of the dose rate on hypoxic cells. This raises another question, as the number of ROS increases proportionally with the dose rate both on aerobic and hypoxic cells, while the cell SF was dose rate-dependent only in hypoxic cells. We hypothesize that this is due to a lack of repair from DNA damage under hypoxic conditions. We considered the possible involvement of DNA DSB repair by acquiring time course data of the number of γH2AX foci following irradiation. From Figure 6(a), it is evident that the number of DSBs does not change significantly 24 h after irradiation under hypoxic conditions. In contrast, from Figure 6(b), it is obvious that the number of DSBs significantly decreases after 24 h of irradiation under aerobic conditions. These results support our hypothesis, whereby the aerobic cells are damaged by ROS resulting in DNA DSBs, but some of the damaged DNA may be repaired due to the existing oxygen. For the hypoxic cells however, repair of DNA damage is limited as previously reported in other research (35). It clarifies why

we could see the dose rate effect for induced ROS with both aerobic and hypoxic cells, but could not see the dose rate effect for cell survival with aerobic cells. This interesting result is also supported by Kumareswaran et al., who observed the decrease of γH2AX foci after the irradiation only in anoxic human fibroblasts (36). We hypothesize that the temporal DNA damage observed in the control group, is caused by physical damage during the transference of cells between facilities, and not fatal error, since we could observe the normal cell proliferation after 24 h as seen in Figure 7. Moreover, the amount of γH2AX foci is higher after 1 h compared to 24 h in the control group. It could be explained that the cells at 1 h were at the logarithmic-growth phase, and high cell proliferation triggered the high amount of γH2AX foci, whereas the cells at 24 h were at the stationary phase, hence cell proliferation is almost stable and so the amount of γH2AX foci was lower (37).

For the hypoxic cells, we suggest that irradiation using high dose rates with FFF beams results in increased production of ROS, which in turn induces a greater number of DNA DSBs without adequate repair of

DNA damage. Therefore, we conclude that antitumor activity observed in hypoxic cells is dose rate-dependent.

In our experiments, we pronominally irradiated cells with 2 Gy, which is the prescribed dose in conventional radiation therapy. As mentioned earlier, FFF beams can be used more efficiently in SBRT to reduce the treatment time, and this is already being implemented in many clinics. Numerous reports regarding FFF beams suggest using a prescribed dose of 8 Gy and above. Kretschmer et al. (38) reported that FFF beams may be used in conventional radiation therapy and concluded that FFF beams may also be feasible for use in 3D conventional radiation therapy. Zhang et al. (39) described the use of FFF beams in intensity modulated radiotherapy for upper thoracic carcinoma of the esophagus, which helped in better lung-sparing compared to treatment using flattening filter. Thus, the use of the prescribed dose of 2 Gy in our experiments is appropriate to further investigate the possibility of using FFF beams in conventional radiation therapy.

Our study clearly demonstrates that the antitumor activity in hypoxic cells exhibits significant differences in a dose rate-dependent manner by using FFF beams. We also showed that hypoxic cells were more resistant to radiation compared to aerobic cells by using FFF beams. Increasing the dose rate resulted in the generation of ROS that trigger DNA DSBs, thus, a reduction in cell survival was observed. Based on the findings of our study, we conclude that the usage of a high dose rate in FFF beams may provide superior tumor control in conventional radiotherapy. Further studies are needed to determine the intracellular interactions that govern the observed phenomenon.

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Figures

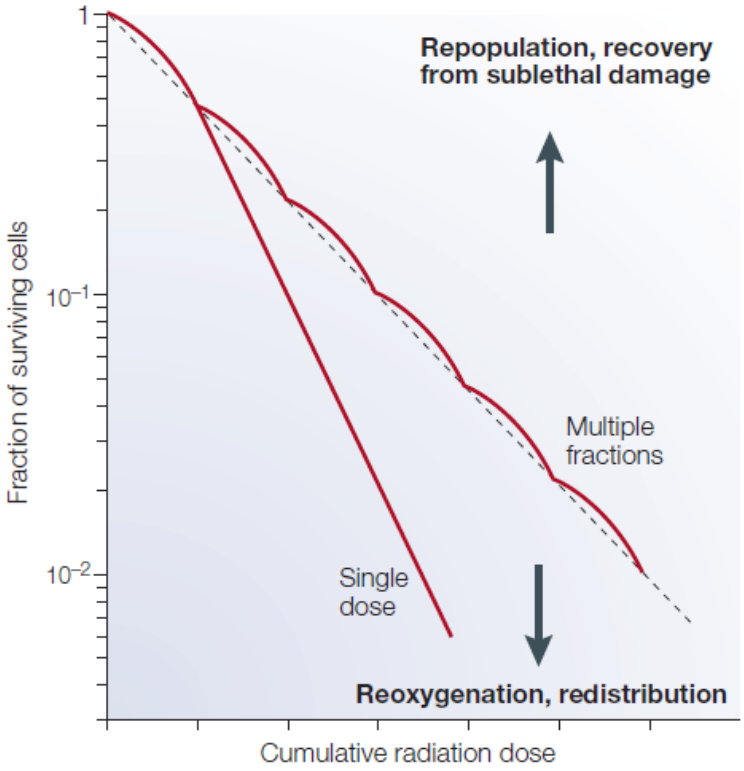


Figure 1: Survival curves and processes that influence response to radiotherapy (figure from reference 2)

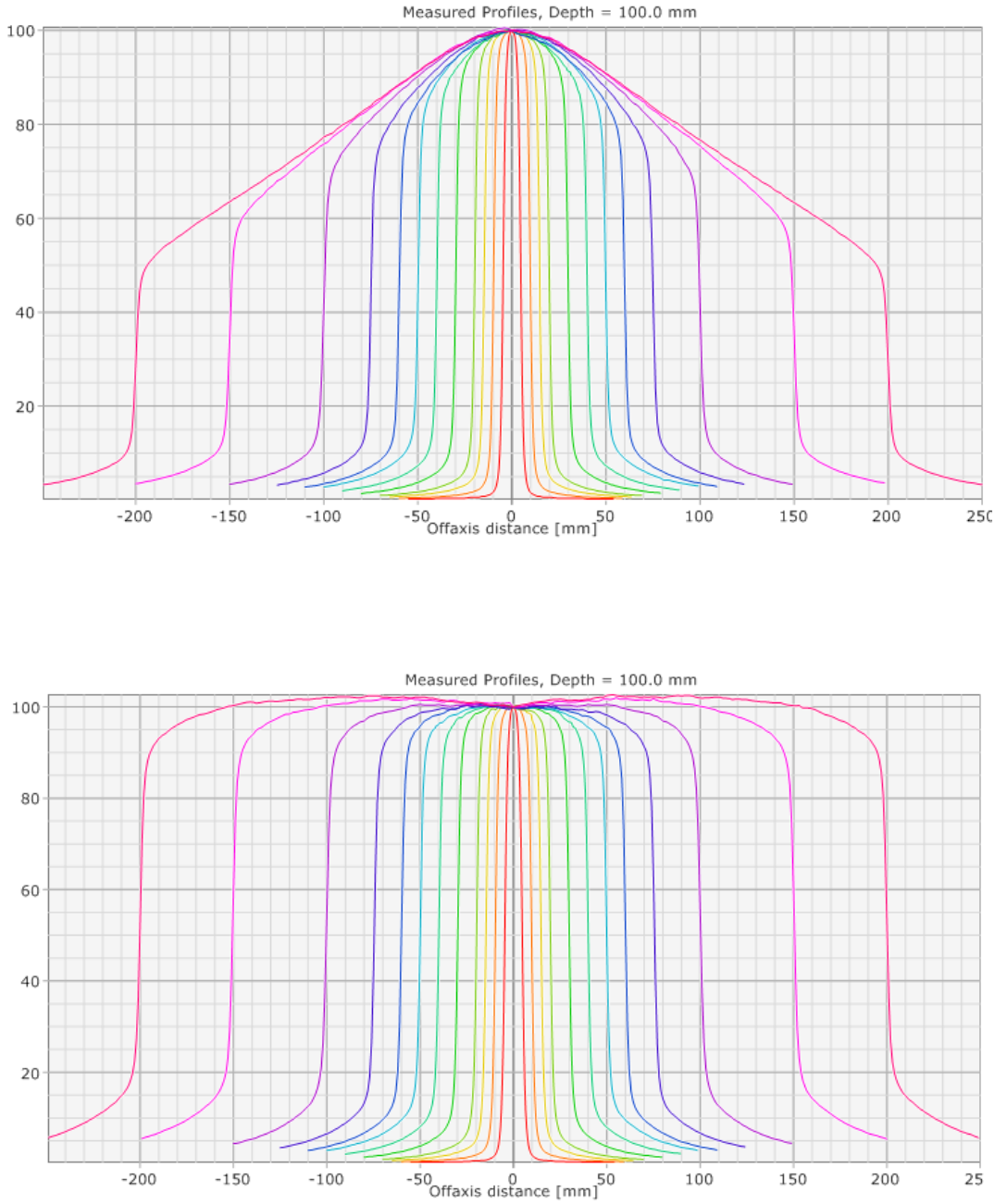


Figure 2: Example of FFF dose profile (Top) and FF dose profile (Bottom)

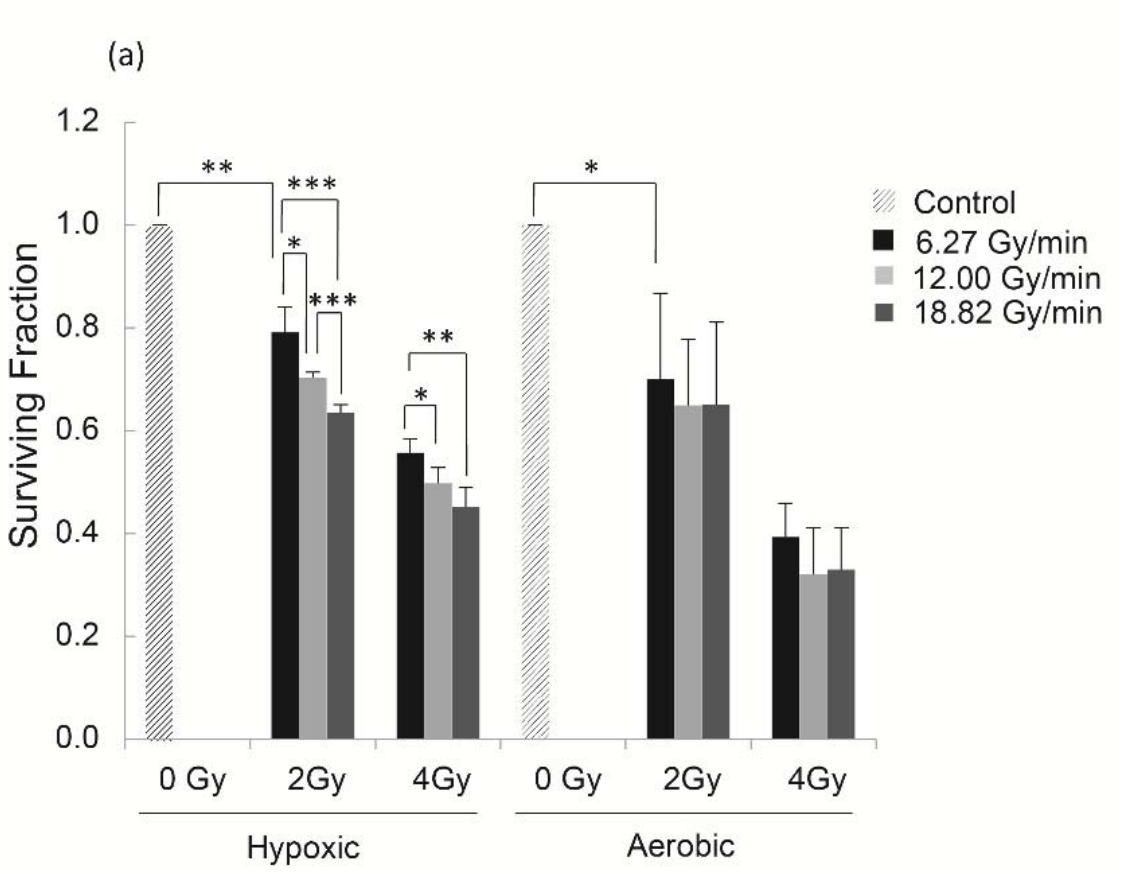


Figure 3(a). SF of EMT6 cells after irradiation plotted as a function of different dose rates (6.27 Gy/min, 12.00 Gy/min, and 18.82 Gy/min) at 2 and 4 Gy. The error bars represent SD calculated for three independent measurements. *p<0.05, **p<0.01, ***p<0.005

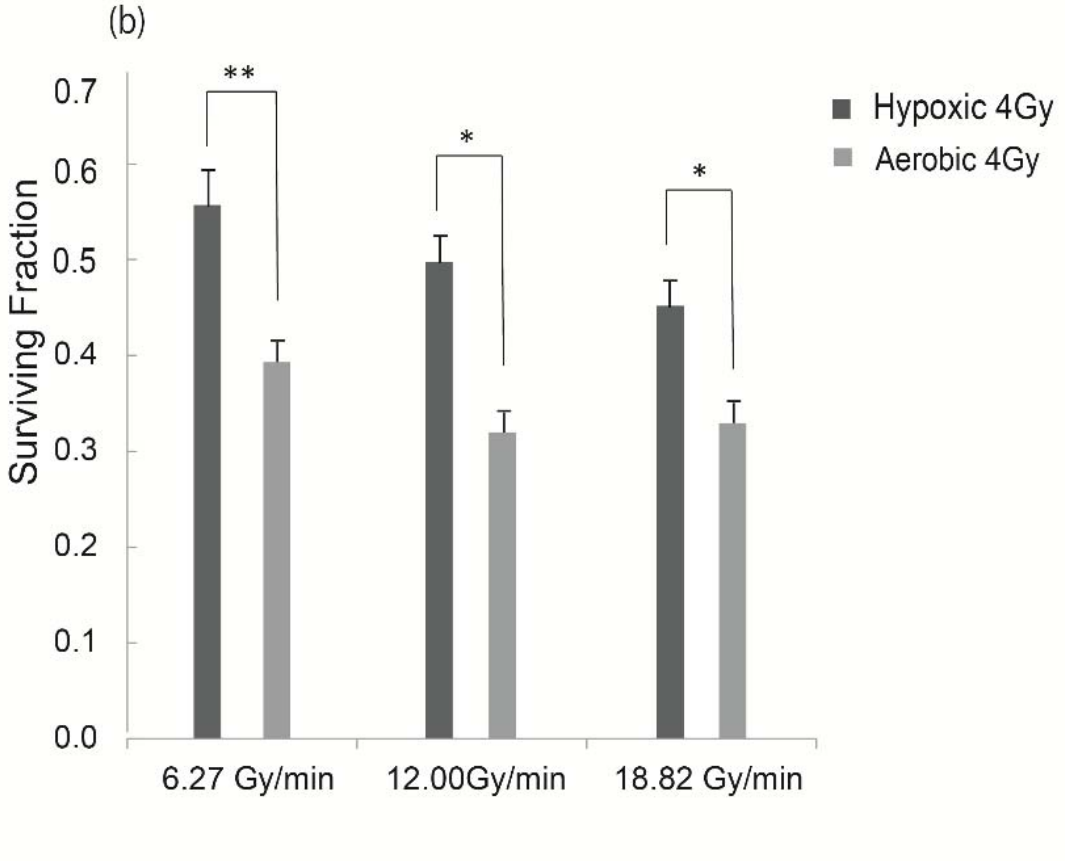


Figure 3(b). The differences in SF between hypoxic and aerobic cells as a function of different dose rates at 4 Gy. The error bars represent SD calculated for three independent measurements. **p<0.01, ***p<0.005

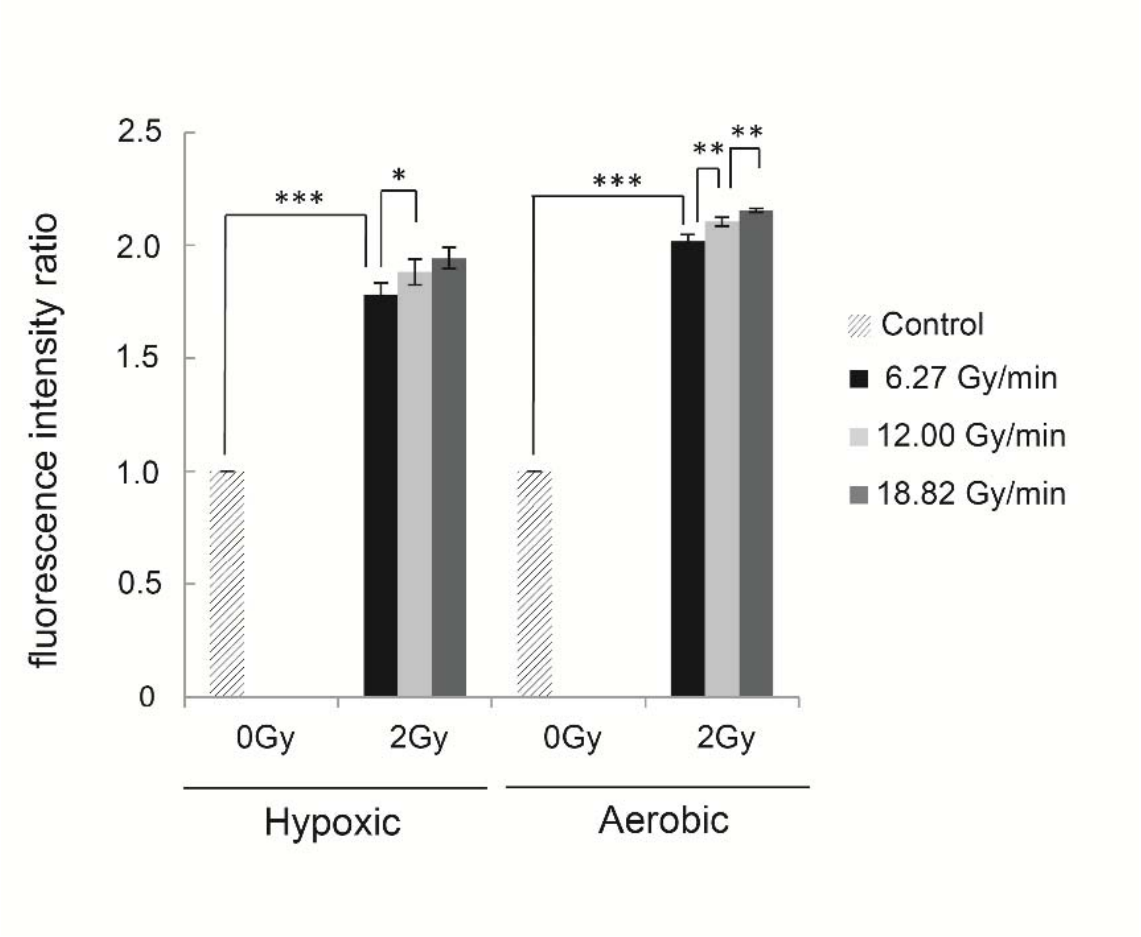


Figure 4. Mean ROS values measured using APF fluorescence shown as a function of dose rate in hypoxic and aerobic cells at 1 h following irradiation at 2 Gy. The error bars represent SD calculated for three independent measurements. *p<0.05, **p<0.01, ***p<0.005

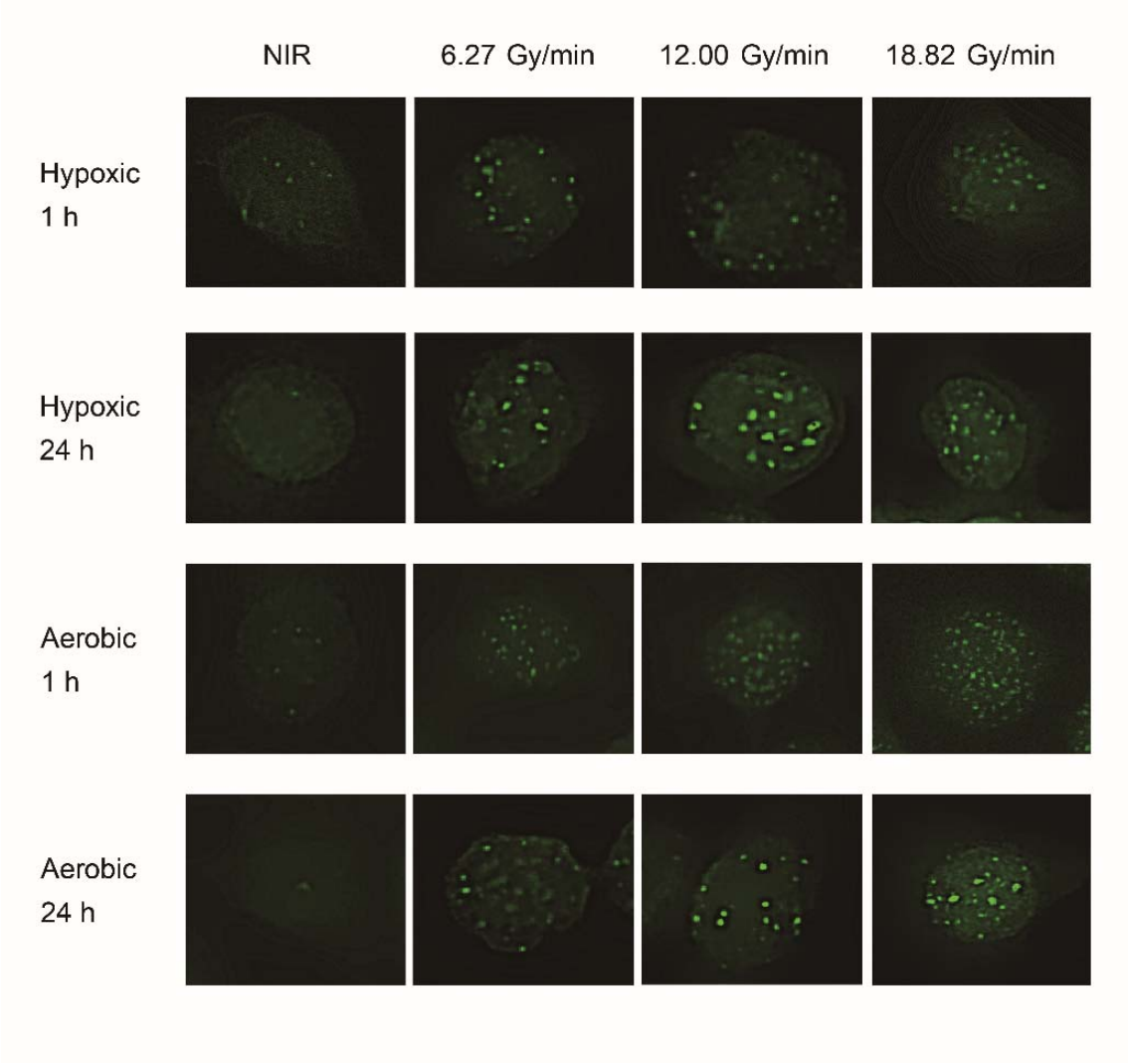


Figure 5. Cell lines under hypoxic and aerobic conditions showing γH2AX foci indicating DNA DSBs with (a) no irradiation (NIR), and 2 Gy irradiation at (b) 6.27 Gy/min, (c) 12.00 Gy/min, and (d) 18.82 Gy/min after 1 h and 24 h of irradiation.

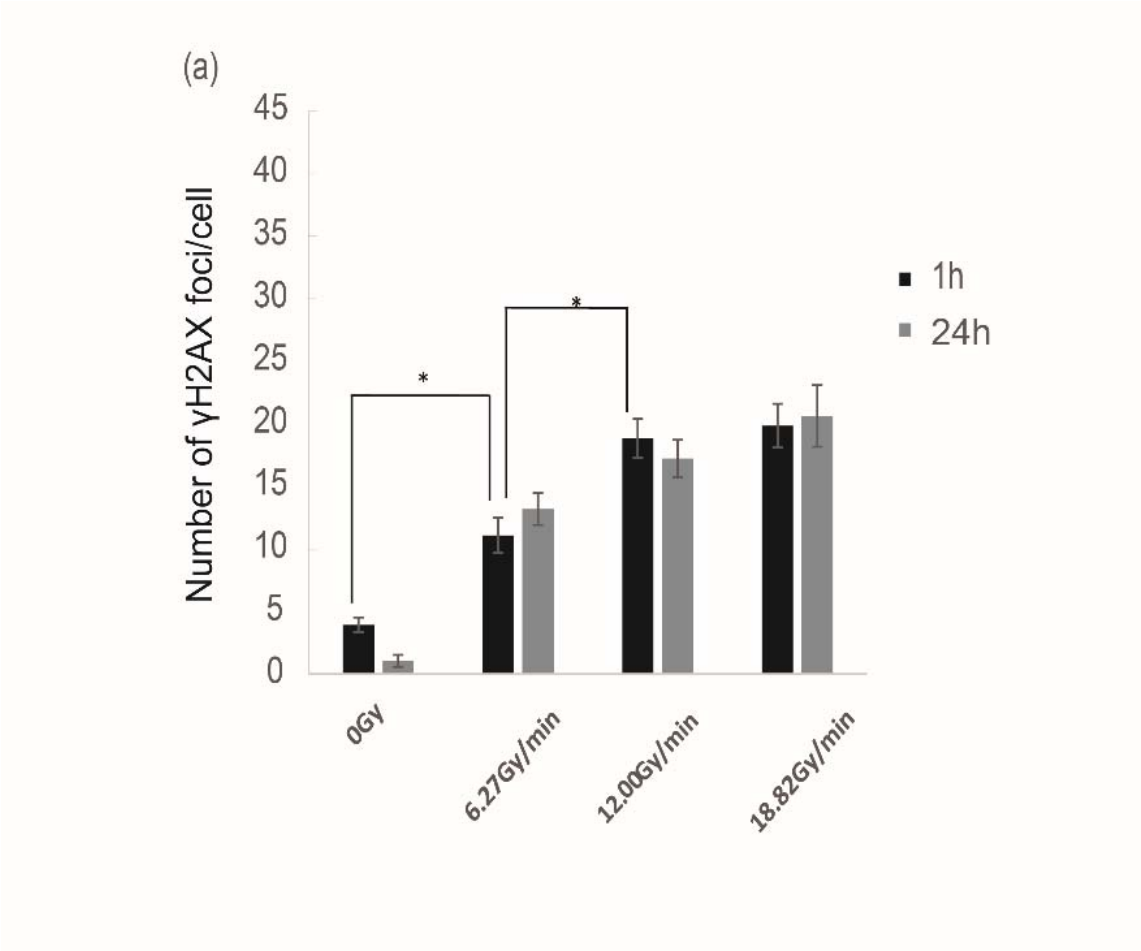


Figure 6(a). Overall mean number of γH2AX foci following 2 Gy irradiation using 6.27 Gy/min, 12.00 Gy/min, and 18.82 Gy/min at 1 h and 24 h for hypoxic cells. The error bars represent SD for three independent measurements. *$p<0.05$

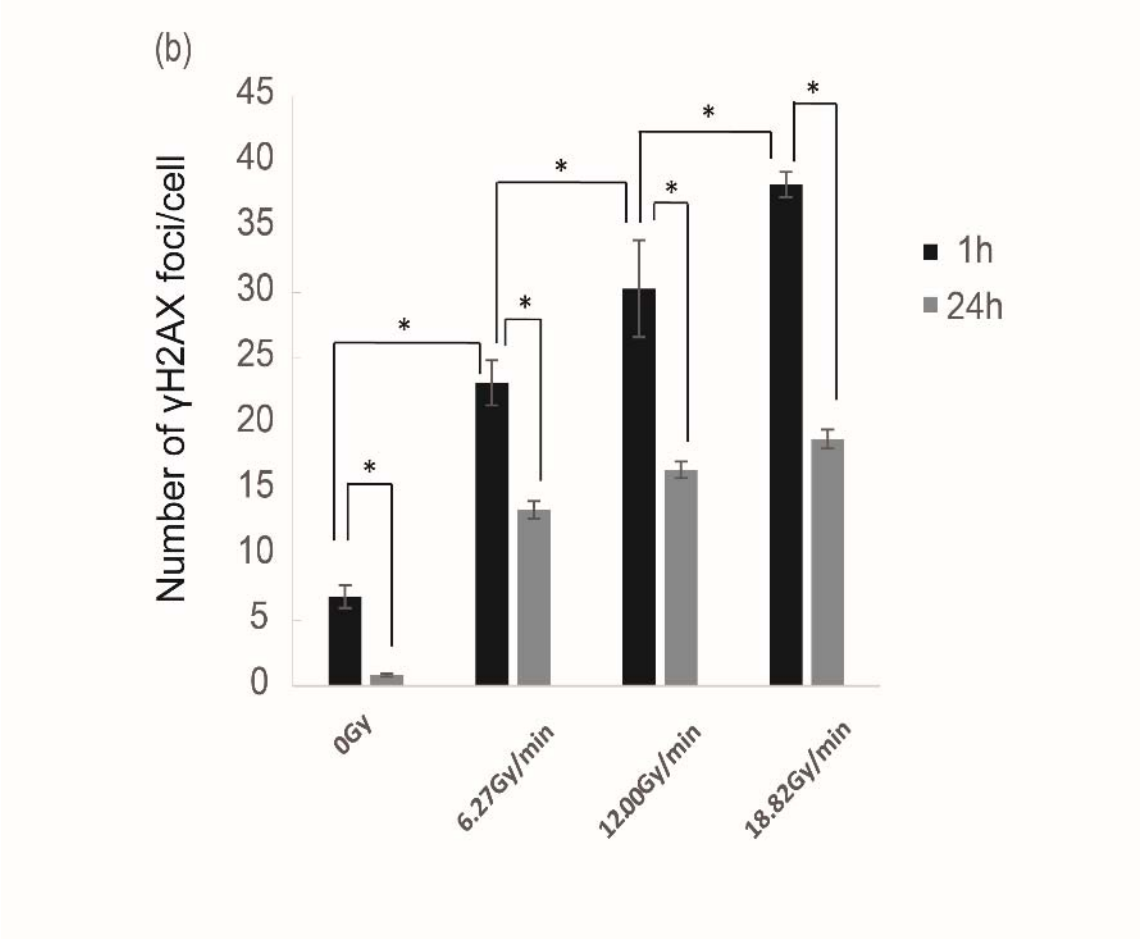


Figure 6(b). Overall mean number of γH2AX foci following 2 Gy irradiation using 6.27 Gy/min, 12.00 Gy/min, and 18.82 Gy/min at 1 h and 24 h for aerobic cells. The error bars represent SD for three independent measurements. *$p<0.05$

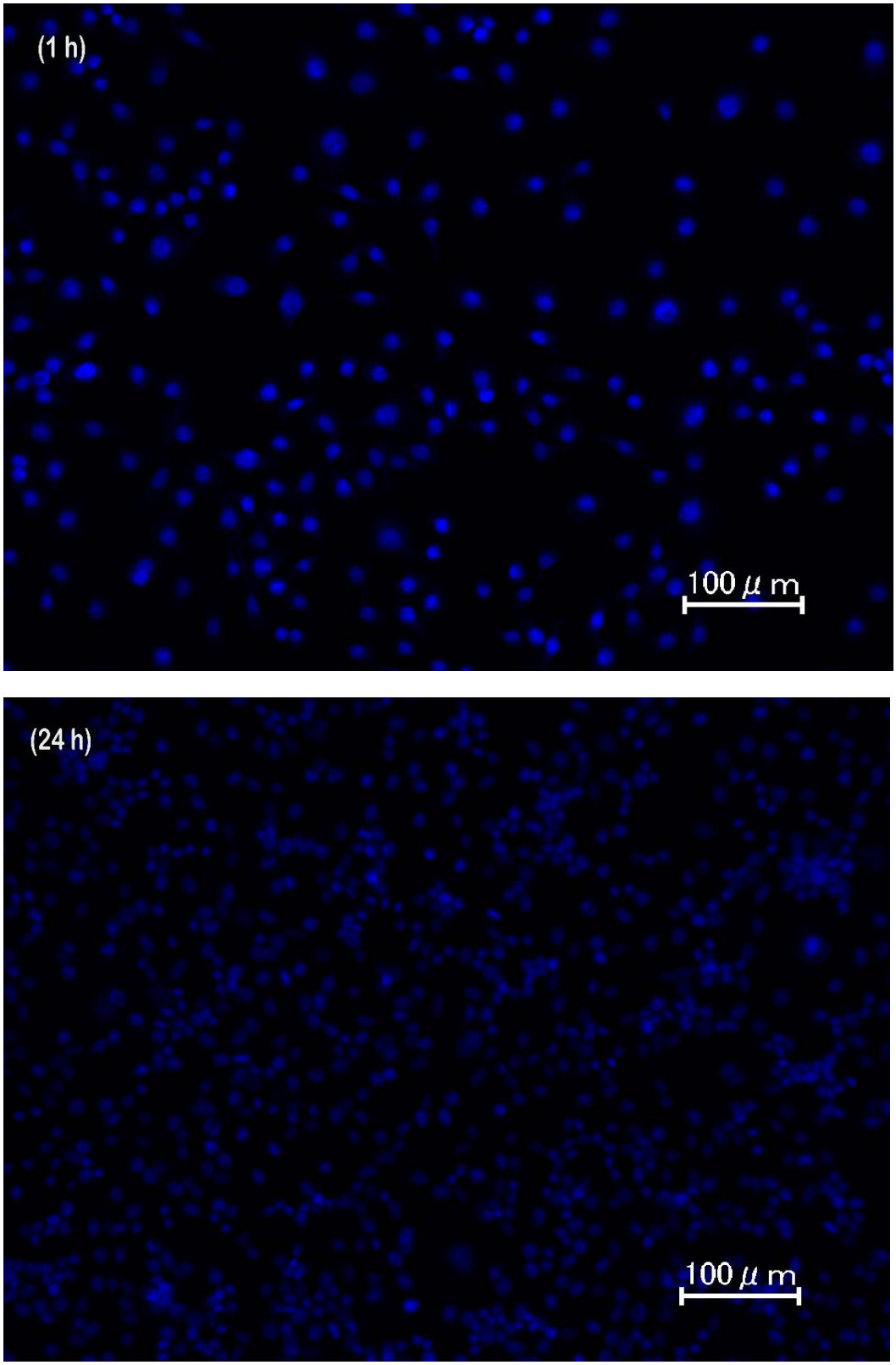


Figure 7. Cells proliferation status in the control group at 1h and 24h.

| Cell status | Dose (Gy) | Dose Rate (Gy/min) | PE | SF | SD |
|---|---|---|---|---|---|
| Hypoxic | 0 | 0.00 | 0.640 | 1.000 | 0.000 |
| | 2 | 6.27 | 0.507 | 0.791 | 0.049 |
| | | 12.00 | 0.450 | 0.703 | 0.011 |
| | | 18.82 | 0.406 | 0.635 | 0.016 |
| | 4 | 6.27 | 0.358 | 0.557 | 0.027 |
| | | 12.00 | 0.320 | 0.497 | 0.031 |
| | | 18.82 | 0.292 | 0.452 | 0.038 |
| Aerobic | 0 | 0.00 | 0.532 | 1.000 | 0.000 |
| | 2 | 6.27 | 0.378 | 0.700 | 0.167 |
| | | 12.00 | 0.345 | 0.649 | 0.129 |
| | | 18.82 | 0.339 | 0.651 | 0.159 |
| | 4 | 6.27 | 0.203 | 0.393 | 0.066 |
| | | 12.00 | 0.180 | 0.320 | 0.091 |
| | | 18.82 | 0.165 | 0.329 | 0.081 |

Table 1. Surviving fraction and plating efficiency of EMT6 cells after irradiation.