THE COMMET ASSAY: A METHOD TO MEASURE DNA DAMAGE INDUCED BY HIGH-ENERGY X-RAY RADIATION

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Abstract. Ionizing radiation can cause different kinds of DNA damage - single- and double-strand breaks, alkali-labile sites, base damage, and crosslinks. Comet assay (an alkaline version of the single cell gel electrophoresis assay) allows us to measure DNA damage at the level of individual cell and in this study was used to evaluate CHO cell line DNA damage induced by ionizing radiation.

Petri dish containing CHO cells were placed inside a PMMA phantom and irradiated with different doses (2-10Gy) of 6MV energy, 3Gy/min dose rate x-ray radiation using Varian linear accelerator. After iradiation within a 2-hour span embedded in low melting point agarose, lysed, in alkaline solution exposed to an electric field(~0.74 V/cm, 300mA), painted with ethidium bromide and visualized using fluorescence microscope. In parallel with the comet assay clonogenic cell viability assay was performed to obtain cell survival curve.

During electrophoresis damaged DNA extends towards anode and form so-called comet tail. The amount of DNA migrated outside of cell boundaries to agarose is proportional to the DNA damage caused by ionizing radiation. DNA damage was evaluated using open source OpenComet software by measuring the relative pixel intensity of comet head and tail fluorescence.

Comet assay results show a linear relationship between radiation dose and DNA damage. This relationship agrees with other studies that performed COMET assay.

After cell viability assay was performed cell survival curve showed higher cell viability compared with a typical cell survival curve, dose was ~40% lower. After closer inspection of irradiation setup it was deduced that the air cavity in the Petri dish caused a sharp drop in the dose distribution, therefore cells were irradiated with a lower dose.

1. Introduction

Exposure to ionizing radiation causes a variety of biological processes in the affected cells, depending on which cells die or are able to survive. The main advantages of Comet assay are that data is collected at the level of the individual cell, the assay can be done using relatively small numbers of cells per sample (<10,000), it has high sensitivity for detecting DNA damage and virtually any eukaryotic cell population is suitable for analysis[1].

Clonogenic cell survival is a basic tool for the study of radiation effects. Much of the information that has been generated by the effect of radiation on mammalian cells has been obtained from clonogenic cell survival assays [2]. A cell that is not reproductively dead and has to retain the capacity to divide and proliferate indefinitely can produce a large clone or a large colony of cells and is the referred as "clonogenic". The ability of a single cell to grow into a large colony that can be visualized with a human eye is a proof that a cell has retained its capacity to reproduce. The loss of this ability after irradiation procedure is described by the dose-survival curve.

2. Materials and methods

2.1. Plating

CHO cell line is an attached cell line, therefore trypsinisation is used to remove cells from plate. Growing medium is removed, cells washed with phosphate buffered saline(PBS) and incubated with a 2ml 0.05% trypsin/EDTA solution for 3 minutes. Once cells become detached, growth medium containing fetal bovine serum(FBS) is added. FBS is used to neutralize trypsin. The cells are detached by pipetting up and down. Hemocytometer is used to count cells. 10^5 cells were plated in 20 dishes, 4 dishes were used for unirradiated control.

400 cells were plated after irradiation for clonogenic cell viability assay using same procedure. Since viability assay is done by counting clones optically plating density must not be too high or clones will coalesce and counting of single colonies will become impossible.

2.2. Irradiation procedure

Irradiation was done using linear accelerator Varian Clinac DMX, using 6 MeV energy and 3 Gy/min dose rate. The irradiation dose varied between 2 Gy and 10 Gy. Field size 10x10 cm.

Petri dish(35x10 mm) with cells was placed in a round 35x10 mm hole in 30x30x11 cm PMMA plastic phantom, the base of the dish with attached cells was at 6cm depth. Dose modeling done with Varian Aria AAA algorithm revealed that 6cm is also the depth of 100% isodose.

2.3. Comet method

After irradiation and within 2-hour span cells were embedded in agarose slides. For that cells were removed from Petri dishes using trypsinization, 10^4 cell were counted from each dish. These cells then were mixed with 75µL, 37˚C, 0.5% low melting point agarose(LMPA)/PBS solution and plated onto slides covered with normal melting agarose(NMA), coverslip placed and left for 5min for LMPA to harden. Coverslip then removed and slides placed in lysing solution(pH = 10) and left in a 4˚C refrigerator for 24h.

After lysing slides are placed in an electrophoresis reservoir with electrophoresis buffer(ph>13) and left in alkaline buffer for 20 minutes to allow for unwinding of DNA. Then slides were exposed to an electric field(~0,74 V/cm, 300mA) for 30 minutes. Electrophoresis at high pH results in structures resembling comets - damaged DNA extends towards anode and forms so-called comet tail. The amount of DNA migrated outside of cell boundaries to agarose is proportional to the DNA damage caused by ionizing radiation. After electrophoresis alkaline is neutralized by drop wise coating them with neutralization buffer.

Slides then are stained with 80 µL of 1X ethidium bromide and visualized using fuorescence microscope under green light(510nm). The amount of DNA in a comets heads and tails are analyzed by capturing images of cells and using OpenComet software.

3. Results

After counting clones, plating efficiency (PE) and survival fraction (SF) can be calculated using the following equations. Average the three dishes colony counts for each dose and divide the mean by the number of cells plated. This will give the PE [3]:

$$PE = \frac{Number\ of\ colonies\ counted}{Number\ o\ cells\ plated} \cdot 100\% \quad (1)$$

Following determination of PE, calculate the fraction of cells surviving. First, normalize all the plating efficiencies of the irradiated samples to that of the unirradiated control dishes, considering that to be 100%. The surviving fraction (SF) is determined by dividing the PE of the irradiated cells by the PE of the controls, and then multiplying by 100 [4]:

$$SF = \frac{PE\ of\ treated\ sample}{PE\ o\ control} \cdot 100\% \quad (2)$$

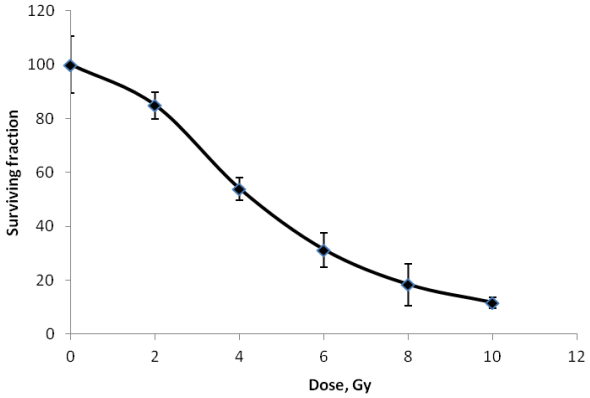

Fig. 1. – Cell survival curve after irradiation. (Error bars: ± 1 SD)

In Figure 1 we see that cell survival of cells decreased by increasing dose. Also, cell surviving curve show higher cell viability compared with a typical cell survival curve[4], as if a dose was ~40% lower. After closer inspection of irradiation setup it was deduced that the air cavity in the Petri dish caused lower dose absorption in and around air cavity region, therefore cells were irradiated with lower dose. Algorithm used to model dose distribution in PMMA phantom is not suitable to accurately calculate dose distribution around air cavities. Other studies using more accurate dose modeling algorithms show that isodose drop in and around 1cm air cavity region can be up to 40%[5].

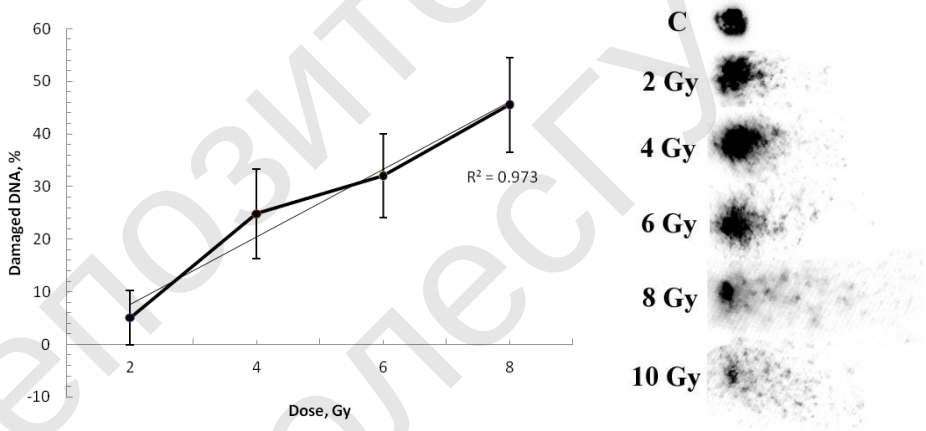

Fig. 2. – Comet assay results (Error bars: ± 1 SD) and examples of comets corresponding absorbed dose. With increasing dose the amount of DNA in the comet head decreases and the amount of DNA in tail increases(C stands for control).

In Figure 2, we see the amount of damaged DNA increases with absorbed dose and correlation is linear. These results agree with other studies[6]. It is also seen that control has no comet tail, therefore there is no damaged DNA that's been affected by electric field. On the other end of the spectrum, cells that absorbed 10Gy dose has almost no comet head, most of the DNA is in the tail and therefore were not suitable for analysis with OpenComet software.

4. Conclusions. Comet assay is a useful tool to measure the amount of DNA damage done to cell culture by ionizing radiation. Measured DNA damage correlates linearly with absorbed dose.

Clonogenic assay suggests that absorbed dose was lower compared with other cell survival curves. The main cause of this is air cavity in Petri dish that lovers absorbed dose in and around air cavity.

References

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