Effect of ionizing radiation on rat peritoneal macrophages

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Abstract **This study aims to evaluate the survival rate of peritoneal rat macrophages after exposure to 3 & 6 Gy ionizing radiation fields, respectively. Cells were cultured in multiwell plates (37 ºC, 5% CO_2), separated in 3 groups: GI–control; GII-cells irradiated with 3 Gy; GIII–cells irradiated with 6 Gy, using a 4 MeV particle accelerator. After irradiation cells were incubated for 2h30 = T0, 13 h = T1, 20 h = T2, 36 h = T3, 70 h = T4, 90 h = T5 post-irradiation. To evaluate the cell's viability the colorimetric MTT test has been used and colour intensity was measured by microELISA. Visual control was done with a contrast phase optical microscope. Irradiation with 6 Gy is the most harmful for these cells, showing death of the majority of them. 3 Gy is also aggressive but enables a higher survival rate. Time is also an important factor to which cell death is proportional. Our results show a decrease of living cells with time and higher doses.**

I. INTRODUCTION

Ionizing radiation is any radiation with enough energy to ionize atoms and molecules. It can harm cells and affect genetic material, DNA, causing serious diseases (e.g. cancer) and eventually death. Alpha particles, electrons and positrons (beta particles), gamma rays and neutrons are examples of ionizing radiation.

The effect of radiation upon cells is conditioned by several factors, mainly: their differentiation level (more differentiated cells are more radioresistant), the phase of the cell cycle (the most important biological stress of a cell is its need to divide, in the medium and late stages of the S phase cells are more resistant to radiation) and their metabolic activity (more active cells are more radiosensitive). This is stated by Bergonie & Tribondeau law, modified by Ancel & Vitemberg, which concludes that the fetus is more radiosensitive. Radiosensitivity depends also upon DNA repairing efficiency by the cell and action of other chemical agents [1].

X-rays have been discovered in 1895 by William Roentgen. They have a wave length between 0.03 nm - 3 nm, being highly energetic (1 keV - 100 keV). This energy range is able to extract electrons from atoms (ionization), inducing chemical changes in molecules. As any ionizing radiation, X-rays have high frequency and can be dangerous [1][2].

Proliferation and cellular viability can be studied using different methods. A colorimetric assay has been chosen: the MTT [3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyl tetrazolium bromide] test. MTT is a tetrazolium water soluble salt, which is converted into a lilac/bluish insoluble product due to the tetrazolium ring cleavage by mitochondrial dehydrogenases of metabolic active cells. Cell membranes are impermeable to this transformation product, being stored inside alive and healthy cells. It is a fast, precise, reproducible, fiable and non-using radioactive markers method. Afterwards an acid solubilizing solution is used to kill and disrupt the cells, dissolving the coloured formed product [3]. The absorbance of this solution is then quantified by spectrophotometry with a microELISA reader [4]. This procedure enables the comparison between samples and a standard.

II. MATERIALS & METHODS

A. Macrophages isolation

Wistar rats, males or females two months old, were previously sacrificed by anæsthetic overdose. Peritoneal macrophages were collected according the usual protocol [5]. The animal was placed inside a laminar flow chamber (Nuaire Biological Safety Cabinet, Class II, Type II Z, Plymouth, MN, USA) in dorsal decubitus. After abdominal skin disinfection with 75% alcohol, the abdomen was exposed without lesion of the abdominal wall. Using a 25 G × ½ needle, adapted to a 10 mL syringe, ±40 mL of a phosphate buffer saline (PBS) 0.15 M, pH 7.2 (8 g NaCl, 0.2 g KCl, 2.9 g $Na_2HPO_4 \cdot 12H_2O$, 0.2 g KH_2PO_4 and bidestiled H_2O up to 1000 mL) were injected without puncturing any abdominal organ. After smooth abdominal massage during 1 - 2 min, the cell suspension of resident peritoneal macrophage was collected (8 to 10 mL) using a 2 - 5 mL syringe connected to a 19 G × 1 needle, to ice cooled Falcon sterile tubes (Falcon plastics, Los Angeles, *CA, USA*).

The peritoneal exudate has been centrifuged at 1100 rpm, during 10 min at 4º C. The pellet has been resuspended

with 3 mL of RPMI 1640 culture medium (Hyclone Cat. No. SH30027.02), previously supplemented with inactive 10% FCS (Gibco B21, BRL, Paisley, Scotland, ref. 19 270), L-glutamine 1% (Sigma-Aldrich, St Louis, Missouri, USA, ref. G-7513), $NaHCO_3$ and peniciline-streptomicine 100 µg/mL (Sigma-Aldrich, St Louis, Missouri, USA, ref. P-4458).

The number of cells in our suspension was determined by counting in a Neubauer chamber (depth: 0.1 mm/0.0025 mm^2, HBG, Berlin, Germany), after (1:1) dilution with Trypan Blue 0.4% (Sigma-Aldrich, St. Louis, Missouri, USA, ref. T-8154) [5].

B. Multiwell plate culture of peritoneal rat macrophages

Cells were placed in sterile multiwell culture plates (6 wells), with the selected cellular density, and incubated at 37º C in an appropriate incubator with a 5% CO_2 flow for 24 h (Nuaire IR autoflow CO_2 water-jacketed Incubator, Plymouth MN, USA). After this incubation period non-adherent cells were removed washing 3 times with a sterile PBS solution (2 mL).

All material and reagents were sterilized by autoclaving (120º C, 20 min); the culture medium was sterilized by filtration, with <0.22 µm filters (Schleider & Schuell ME 24/21, Berlin, Germany).

C. Exposition to different irradiation doses

After isolation and culture, macrophages were irradiated in a particle accelerator (X-ray Varian-Clinac® 600C, 4 MeV) with two different doses (3 and 6 Gy, respectively) (Instituto Português de Oncologia, Coimbra). The sealed multiwell plates were irradiated at 100 cm from the source, using a 20×20 field, diverging from the focus.

Our experiment proceeded then with the three samples: control (non-irradiated plate), 3 Gy and 6 Gy irradiated plates.

Adherent cells were detached from the wells using 300 mL of a 0.5% trypsine solution (2 min in the incubator + optical microscope control) and re-incubated in triplicates in a sterile 96 well plate (Well cell culture cluster with flat bottom, Costar, Corning, NY, USA, refª 3596), in the previously referred culture medium at 37º C and 5% CO_2 atmosphere for 2.5 h, according to the usual protocol [5].

Proliferation and cytotoxycity tests were performed at: 2h30 = T0, 13 h = T1, 20 h = T2, 36 h = T3, 70 h = T4 post-irradiation. 90 h post-irradiation the same test has been performed in the remaining cells of the three mother plates (their media have also been renewed when necessary, according to the usual medium colour change sign).

D. Proliferation and cytotoxycity tests for macrophages

In order to evaluate cell viability after irradiation, the MTT colorimetric test [3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma-aldrich, St. Louis, Missouri, USA, ref. M-2128) has been used.

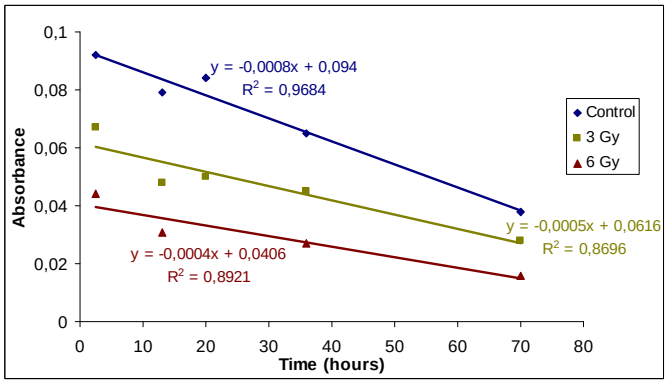
The MTT solution (5 mg/mL in PBS) has been sterilized by filtration, a technique which also removes small amounts of insoluble residues.

After removing the old culture medium, macrophages were incubated with 270 µL of fresh RPMI and 30 µL of MTT solution, in the previously described condtions (37º C, 5% CO_2) during 2 - 3 h. At this stage, the prior solution was removed and isopropanol acid (300 µL HCl 0,04 N in isopropanol) was added to burst the cells and dissolve the coloured reduction products of the MTT reaction. MicroELISA SLT (Labinstruments Ges m b H, Salzburg, Austria) reading was done 15 min after, to ensure that all formazan crystals were dissolved, at 570 nm with a reference filter of 620 nm. Meanwhile some digital pictures were obtained using a contrast phase optical microscope and a digital camera Nikon coolpix 5400.

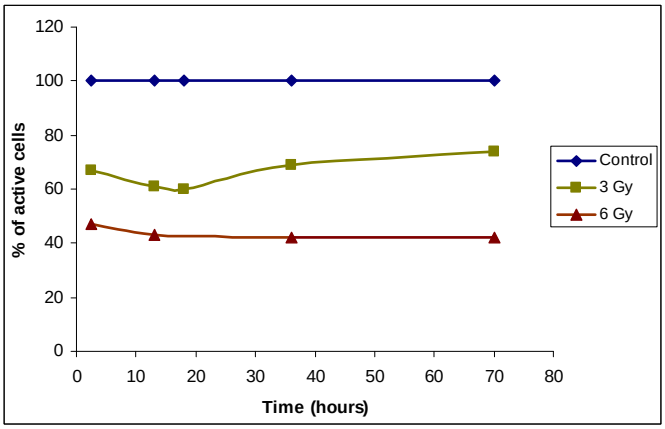
III. RESULTS

Table 1: Absorbance results and corresponding alive cell % for the three samples.

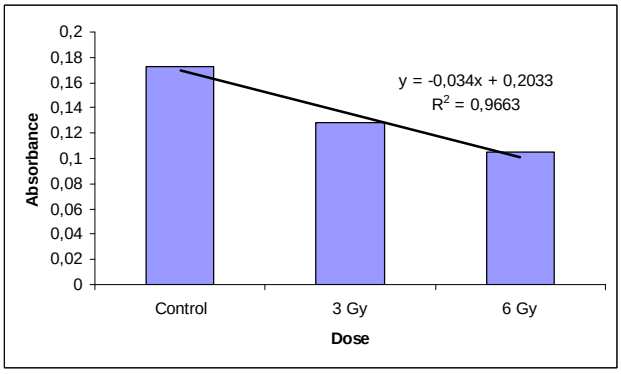
	Control		3 Gy		6 Gy	
Time (h)	Abs.	cell %	Abs.	cell %	Abs.	cell %
2.5	0.092	100	0.067	67	0.044	47
13	0.079	100	0.048	61	0.031	43
20	0.084	100	0.050	60	0.046	55
36	0.065	100	0.045	69	0.027	42
70	0.038	100	0.028	74	0.016	42
90	0.173	100	0.128	74	0.105	61



Graphic 1: Absorbance along time for the three samples.



Graphic 2: % of active cells along time for the three samples.



Graphic 3: Absorbance at T5 = 90 h for the three mother plates remaining cells.

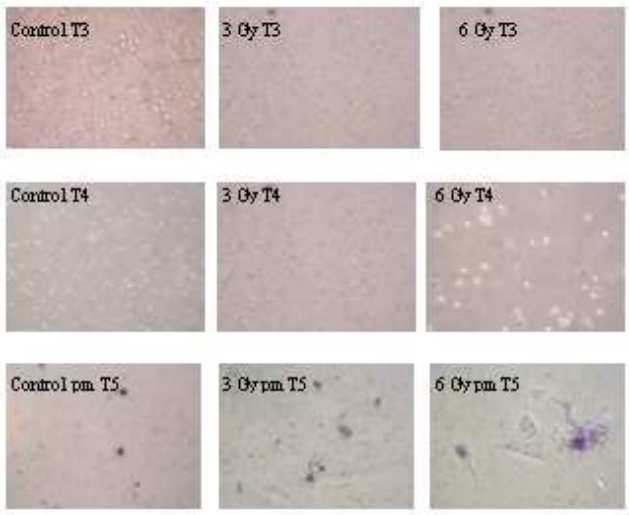


Figure 1: Digital pictures obtained using a contrast phase optical microscope at different times for the three samples (mother plates = pm).

IV. DISCUSSION

The non-irradiated sample (control) was the standard to compare the irradiated macrophages cultures. Control is always shown as 100% of maximum active cells for each time.

Macrophages show an adapting/reactive stage to radiation effects (toxic effects). For 3 Gy one can observe an increase in the number of active macrophages around 20 h post-irradiation, and they stabilize at 74% for 70 h. Due to a higher dose, 6 Gy, this reaction is slower, showing some recovery around 70 h (increase in the % of active cells – Graphic 2).

For 6 Gy, due to the very low correlation index (R^2=0.468) indicating a bad fitting, the value at 20 h has been discarded. It accounts for a less accurate technical procedure: the high blue colour concentration was due to volume changes and not to a higher number of viable cells.

During the first hours post-irradiation there has been cell death both for 3 and 6 Gy, showing a higher death incidence for 6 Gy. The lines in Graphic 1 are not parallel, nevertheless the slope is not much different. This indicates that the experiment evolved as expected. An absorbance decrease with time is observed in all cases, meaning that the number of viable cells decreases with time, due to macrophages particular culture characteristics.

It is interesting to note (Table 1) that macrophages irradiated with 6 Gy at 90 h post-irradiation present a significant recovery (42% to 61%) when comparing with macrophages irradiated with 3 Gy, which maintained a 74% viability. This sample had shown an earlier recovery and then they stabilized.

As shown in Graphic 3 (absorbance values for the mother plates), the negative line slope traduces the absorbance decrease (number of active cells) with increasing irradiation dose. In spite of significant recovery, both for 3 and 6 Gy, cells irradiated with higher dose do not attain, at 90 h post-irradiation, such a good recovery. Their survival rate never reaches the values obtained for the control plate due to the toxic effects of irradiation in our experiment conditions. The digital photographic images obtained comprove our statements (Figure 1). The active cells engulf the MTT dye and metabolize it, which changes its yellowish colour to a lilac/blue colour. It is important to refer that the darker the lilac/bluish tone the more active cells are. Comparing photos of the control sample and 6 Gy, we can see groups of coloured cells but with different intensities of blue colour. This can be due to a less activity of 6 Gy irradiated macrophages. Overlapping of cells (in the control sample) would not give this kind of images.

Another feature shown in the photos (Figure 1), is the bigger size of 6 Gy irradiated macrophages. These cells are attempting to react to adverse conditions, by opposition to normal cells of the control sample which maintain their normal morphology. As expected, macrophages irradiated with 3 Gy are also bigger than in the control ones, but smaller than the ones irradiated with 6 Gy.

V. CONCLUSIONS

Macrophages are sensitive cells easy to obtain at a reduced price. The MTT test is a standard method for

proliferative and cytotoxicity viability studies chosen by a large number of research teams.

To a higher radiation dose corresponds a decrease in viable cells. Macrophages try to adapt to induced toxic conditions and they recover to some extent along time. Recovery/adaptation time is longer as the exposition dose increases, never reaching 100%. Nevertheless a higher number of recovered cells may also indicate a possible synergetic group effect. If the number of dying cells is higher there will be more cell debris and toxic metabolites that may impair the stunned cells to recover.

This in vitro experiment using peritoneal rat macrophages enabled some enlightening on radiation effects in vivo.

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