

EVALUATION OF THE EFFECT OF RADIATION LEVELS AND DOSE RATES IN IRRADIATION OF MURINE FIBROBLASTS USED AS A FEEDER LAYER IN THE CULTURE OF HUMAN KERATINOCYTES

Daniele Yoshito¹, Tiago L. Almeida¹, Stefany Plumeri Santin¹ Elizabeth S. R. Somessari¹, Carlos G. Da Silveira¹, Silvana C. Altran², César Isaac², Marisa R. Herson³ and Monica B. Mathor¹

¹ Instituto de Pesquisas Energéticas e Nucleares, IPEN - CNEN/SP
Av. Professor Lineu Prestes 2242
05508-000 São Paulo, SP
daniyoshito@uol.com.br

² Laboratório de Microcirurgia Plástica da Faculdade de Medicina da USP
Av. Dr. Arnaldo 455, 1º andar sala 1363
01246-903 São Paulo, SP

³ Donor Tissue Bank of Victoria
57 - 83 Kavanagh St.
Southbank - Victoria 3006 Australia

ABSTRACT

In 1975, Rheinwald and Green published an effective methodology for obtaining and cultivating human keratinocytes. This methodology consisted of seeding keratinocytes onto a feeder layer composed of lineage 3T3 murine fibroblasts, the proliferation rate of which is then controlled through the action of ionizing radiation. The presence of the feeder layer encourages the development of keratinocyte colonies and their propagation in similar cultures, becoming possible several clinical applications as skin substitutes or wound dressings in situations such as post burn extensive skin loss and other skin disorders. However, good development of these keratinocytes depends on a high quality feeder layer among other factors. In the present work, we evaluated the relationship between radiation levels and dose rates applied to fibroblasts used in construction of feeder layers and the radiation effect on keratinocytes colonies forming efficiency. Results indicate 3T3 lineage murine fibroblasts irradiated with doses varying between 60 and 100 Gy can be used as a feeder layer immediately after irradiation or storage of the irradiated cells in suspension at 4 °C for 24 hours with similar results. The exception is when the irradiation dose rate is 2.75 Gyh⁻¹; in this case, results suggested that the fibroblasts should be used immediately after irradiation.

1. INTRODUCTION

Skin is considered the largest human organ, representing 16% of body weight [8], being an efficient barrier against a variety of physical, chemical, and biological agents, besides participating in homeostatic and physiological regulation processes [9,11]. For such reasons, the occurrence of any damage to its integrity may have an impact in the patient's survival rate, as the case of people that suffered serious burns or extensive, who are susceptible to infections and septicemia due to depression of the immune response [8]. In order to avoid these infections, therapeutic procedures were developed to recover the skin, such as cultivation of keratinocytes.

In 1975, Rheinwald and Green published a methodology for cultivating human keratinocytes, in which irradiated murine fibroblasts were used to form a feeder layer, making possible the lifespan extension of keratinocytes in culture [12, 13]. During the experiment, they modified a murine fibroblast cellular lineage (NIH-3T3), obtaining a lineage called 3T3-J2, which after irradiation was used as a support to keratinocytes culture [10, 12, 13].

The importance of using irradiated fibroblasts as feeder-layer is due to these cells's capacity to synthesize fibronectin, small amounts of Type IV collagen, and laminin [1, 5]. These cells also release growth factors [4, 5] and extracellular matrix proteins into the culture medium, facilitating keratinocytes adhesion and growth [1, 5].

Irradiation of murine fibroblasts is necessary to control their growth so that it is not opposed to the growth of key cells to be cultivated. A dose of 60 Grays (Gy) was set up by Rheinwald and Green as enough to inhibit fibroblast duplication [12, 13].

With the formation of a feeder layers, formed by irradiated murine fibroblasts, it was possible to develop keratinocytes in colonies and propagate the cells in further cultures, allowing confection of transplantable epithelium [3, 6, 7, 8, 10]. However, proper keratinocytes development depends on a good quality feeder layer, among other factors.

In a previous study conducted by our group [2], it was possible to show the physico-morphological variation of this feeder layer, related to the type of culture medium irradiation doses, and storage after irradiation. Continuing that work, this study evaluated the relation between doses and irradiation dose rates employed to produce feeder layers and formed keratinocytes colonies.

2. MATERIAL AND METHODS

2.1. Medium Preparation

Culture medium D10: Dulbecco's Modified Eagle's Medium (DMEM), 10% of bovine calf serum, penicilin (100 U/mL)/streptomycin (100 µg/mL), amphotericin B (2,5 µg/mL) and L-glutamin (4 mM)

Culture medium K-: Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 (2:1) supplemented with 10% of fetal bovine serum, penicilin (100 U/mL), streptomycin (100 µg/mL), amphotericin B (2.5 µg/mL), glutamine (4 mM), adenin (0,18 mM), insulin (5 µg/mL), hydrocortisone (0,4µg/mL), cholera toxin (0,1 nM), triiodothyronin(20 pM).

Culture medium K+: For make K+ it's add Epidermal Growth factor (10 ng/mL).

2.2. Fibroblast Culture

Approximately 1×10^6 murine fibroblasts (CCL-92, ATCC) were defrosted and plated in cell culture flasks, at the density of 2.8×10^3 cells/cm², in culture medium D10, at 37°C, in a humidified atmosphere of 5% of CO₂. When cells reached a 70% 80% confluence rate, were detached from the flask with trypsin (0,05%)/EDTA(0,02%) solution and irradiated, at pre determined doses and doses rate, at room temperature in the Cobalto-60 irradiator (type Gammacell), at the CTR/IPEN nuclear irradiation plant.

2.3. Feeder Layer Preparation

Fibroblasts were irradiated with 60, 70, and 100 Gy doses. For each dose, attenuators of 90%, 70%, 50% were employed, as well as without attenuator. Thus, respective doses rate varied from 0.275 to 2.75 kGy h⁻¹. Irradiated fibroblasts were separated into two parts: one was seeded immediately after irradiation and the other was stored at 4 °C during 24 hours. By employing a multi-well plate of 6 wells (area of 9.62 cm²/well), irradiated cells were seeded in the 3×10^4 cells/cm² density, in K+ medium, and kept at a humid incubator adjusted to 37 °C, with 5% CO₂ atmosphere. After 24 hours, the cells kept at 4 °C were seeded in culture plates and kept in the sterilizer until receiving keratinocytes.

2.4. Keratinocytes Culture

Human keratinocytes were obtained from skin samples donated by the Tissue Bank of the FMUSP Clinical Hospital's Central Institute. The samples were submitted to a cellular dissociation treatment with enzymatic action (Trypsin), in periods of 30 minutes at 37 °C. Obtained keratinocytes were seeded at 2×10^4 cells/cm² density over a feeder layer formed by previously irradiated murine fibroblasts (60 Gy). The keratinocytes/feeder layer set was nourished through a K+ medium, and kept in a humid incubator adjusted to 37 °C, with 5% CO₂ atmosphere. At the sub-confluence of the primary culture, cells were trypsinized with 0.05% Trypsin/0.02% EDTA and seeded in a new feeder layer, which became the secondary culture. At the sub-confluence of the secondary culture, cells were trypsinized, counted, and employed for experiments.

2.5. Colony Forming Efficiency (CFE)

After adhesion of irradiated fibroblasts, 300 keratinocytes were seeded per well. The keratinocyte/feeder layer culture was kept in K+ culture medium. Medium exchange was done at every 48 hours during 14 days. After this period, cultures were colored with Rodamin B and keratinocyte cultures were morphologically analyzed and counted, taking into consideration the number of developing colonies and formation of abortive colonies.

2.6. Statistical Analysis

Graph-Pad Prism, Version 4.0, was used to perform the statistical analysis. As parameters were not distributed normally, statistical analysis was performed by non-parametric method. The Kruskal-Wallis test was used to compare variation of dose rates for 60, 70 and 100 Gy doses. We considered differences to be significant when P values were <0.05 .

3. RESULTS AND DISCUSSION

According to Figure 1A, it can be observed that there was no significant alteration in the amount of keratinocyte colonies obtained by CFE experiments, which had the feeder layer seeded immediately after irradiation, even when different dose rates were employed (0.275; 0.825; 1.375; and 2.75 kGy h⁻¹). These data were confirmed by statistical analysis, which identified no significant difference.

The same behavior does not occur with fibroblasts seeded 24 hours after irradiation (Figure 1B), in which case there is a tendency to decrease the amount of obtained colonies (approximately 20%), with doses of 70 and 100 Gy, when these fibroblasts were irradiated with dose rate of 2.75 kGy h⁻¹ (0% attenuation), but this difference did not achieved statistically significant.

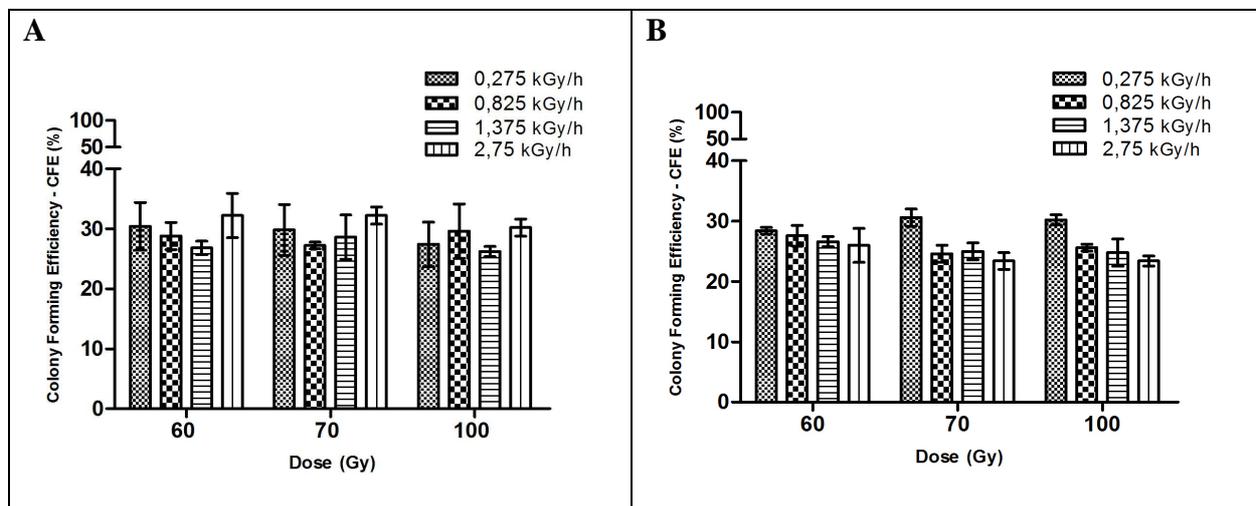


Figure 1. Keratinocyte CFE variation histogram, when seeded with feeder layer formed by fibroblasts irradiated with different doses and dose rates. A) Fibroblasts seeded immediately after irradiation. B) Fibroblasts seeded after 24 hours of irradiation.

It can be verified through Figure 2 histograms that the number of abortive colonies observed in CFE of keratinocytes was homogeneous for all cultures, independent on dose, dose rate, and seedling time. This demonstrates that even if the fibroblasts are irradiated at 100 Gy, they keep presenting their support potential and producing factors that allow keratinocyte development and duplication.

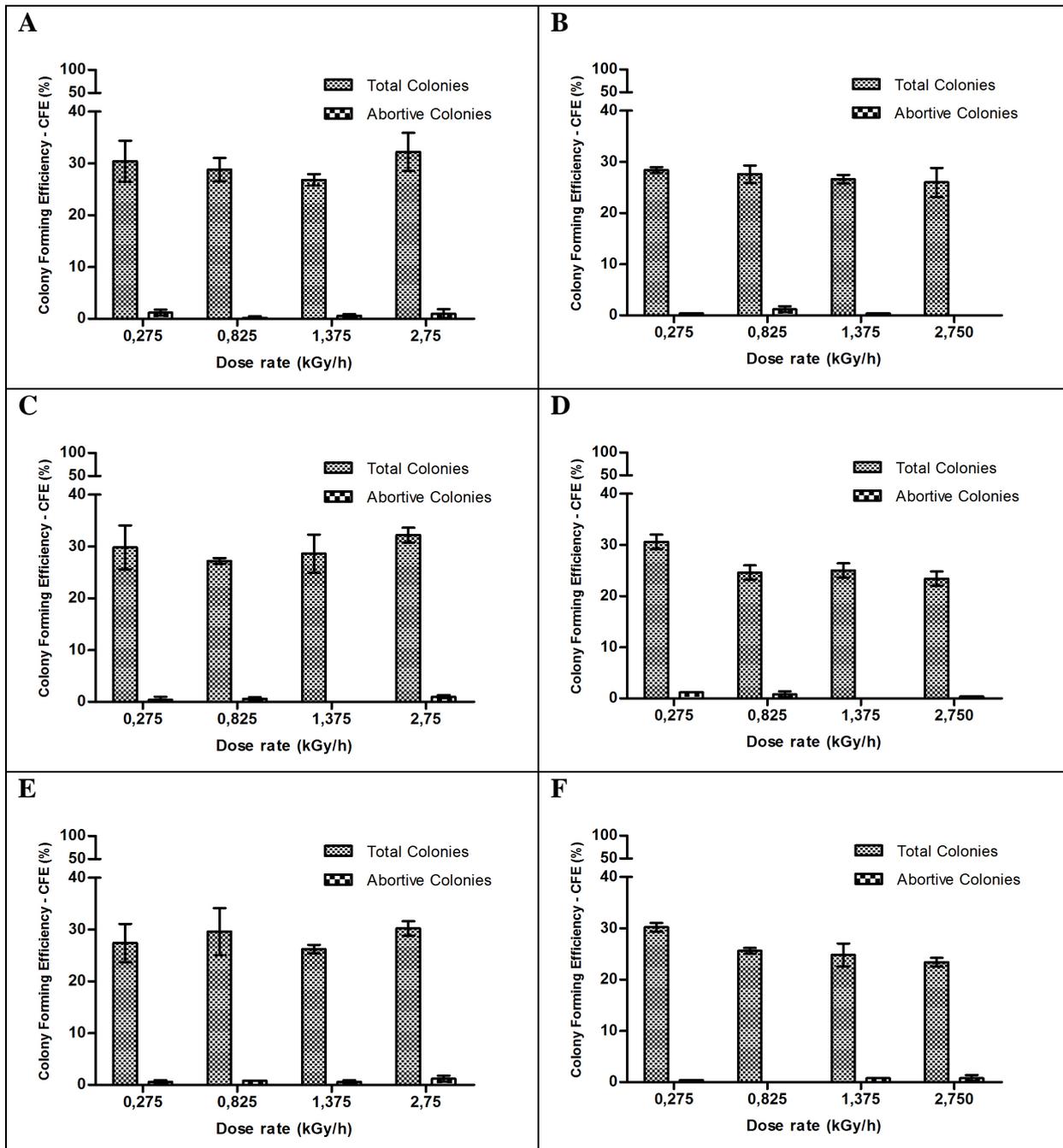


Figure 2. Percentage of abortive colonies, in relation to CFE of total keratinocytes with different dose rates, at doses of 60 Gy (A and B), 70 Gy (C and D), and 100 Gy (E and F). Where: (A, C, and E) fibroblast seeded immediately after irradiation. (B, D, and F) fibroblasts seeded after 24 hours of irradiation

4. CONCLUSIONS

3T3 murine fibroblast lineage (CCL-92, ATCC) can be used as feeder layer, the statistical analysis showed no significant difference when these cells were irradiated with doses varying from 60 to 100 Gy, seeded immediately after irradiation or after 24 hours of storage at 4°C. However, in the case of source dose rate values of 2.75 Gy h⁻¹, it is suggested that these cells be used immediately after irradiation.

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