

IRRADIATED MURINE FIBROBLASTS AS FEEDER LAYER USED IN HUMAN CELL CULTURE

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ABSTRACT

In 1975, Rheinwald and Green published an *in vitro* model for keratinocyte cell cultures in which the use of murine fibroblasts, as a feeder layer was introduced. These cells are modified fibroblasts, which presence render keratinocyte cells to remain proliferative for longer periods of time. This optimization of culture outputs has allowed for several clinical applications of confluent keratinocyte cultures as skin substitutes or wound dressings in situations such as post burn extensive skin loss, loss of oral mucosa, and other skin disorders. Nevertheless, proliferation of fibroblast in co-culture with keratinocytes must be controlled by anti-proliferative measures such as irradiation; at the same time, keratinocytes require specific nutrients in the culture medium, which may interfere with the fibroblast feeder layer viability. Therefore, the thorough understanding of the impact of different issues such as culture media composition, irradiation dose and pre-plating storage conditions of irradiated fibroblast to be used as feeder layer in these co-culture systems is important. In this work, changes as far as viability and proliferative rates of irradiated fibroblasts in culture were evaluated in relation to the type of culture medium used, dose of gama radiation exposure, storage and timing of cell plating post irradiation. Results indicate that the type of culture medium used and time-lag between irradiation, refrigeration and plating of irradiated cells do not have significant impact in culture outcomes. However, the dose of gama radiation administered to the cells may influence the final quality of these cells if to be used as a feeder layer.

1. INTRODUCTION

Our skin is a most importance as a barrier to the environment by hindering the penetration of microorganisms into our bodies, thus preventing several problems and diseases [7,6,9]. This protection may be lost or severely compromised when, for several reasons, this tissue layer is damaged, like in extended burn injuries. Therefore, the challenge of finding the means to substitute skin has been translated into an important field of research. Being the skin mainly constituted by confluent keratinocytes [6,7], one of the obvious possibilities to be explored was the feasibility to establish keratinocyte cell cultures *in vitro*[4]. Until 1975, this type of culture was successfully carried out using a method, which required the plating of keratinocyte cells in high densities, implying in the need for large numbers of cells for establishing the cultures. All considered, keratinocytes retain their proliferative capacity, despite low efficiency rates and the tendency to premature differentiate into disorganized colonies. These aspects evidenced that this methodology could be quite impractical when large expansion of primary cultures, such as in skin replacement, were desired.

In 1975, Rheinwald and Green published an innovative method of an *in vitro* keratinocytes culture, where they used other cells to form a sustentation layer called feeder layer, thus becoming possible the culture of these keratinocytes for longer period [10, 11]. For the use of these cells as sustentation layer, they modified murine fibroblasts cell line (NIH-3T3), arriving at called 3T3-J2 line cell [8,10,11], later deposited in the ATCC under acronym CCL-92. With the formation of this layer was possible the development of uniform keratinocytes colonies, however the proliferation of the fibroblasts must be controlled so that their growth do not overlap the growth of the interest cells. To control these cells growth it was used ionizing radiation [12]. Thus these fibroblasts lose their division capacity leading, in long stated period their lethality [8,10,11]. The dose of 60 Grays (Gy) was praised as enough to inhibit the duplication of these fibroblasts. Besides constituting this layer of sustentation, the irradiated fibroblasts seem to exert an important paper, where they continue to produce nutritional substances and/or growth factors, assisting the keratinocytes in the development of the culture [1,7,11].

Other work shows the importance of the correct irradiated fibroblasts density for the formation of the feeder layer where an exaggerated or insufficient addition of those, disfavors the growth and the sustentation of the keratinocytes [2,10,11]. With the optimization of this type of culture, several clinical applications become possible and therefore verifying the importance of this improved methodology which can in giving scientific support to them to contribute more and more in the direction and the search of new answers for the pathologies and its repairs, such as the case of the skin. [3,10]. The main of this work was the characterization of the fibroblasts (ATCC-CCL-92) as sustentation layer in relation to the type of culture medium, the irradiation dose, storage time before seeding.

2. MATERIALS AND METHODS

2.1 Medium Preparation

Culture medium D10: Dulbecco's Modified Eagle's Médium (DMEM), 10% of bovine calf serum, penicilin (100 U/mL)/streptomycin (100 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 bovine calf serum, penicilin (100 U/mL), streptomycin (100 µg/mL), gentamycin (50 µg/mL), anfotericin B (2.5 µg/mL), glutamine (4 mM), adenine (0,18 mM), insulin (5 µg/mL) , hydrocortisone (0,4µg/mL), cholera toxin (0,1 nM), triiodothyronin (20 pM) For make K+ it's add Epidermal Growth factor (10 ng/mL).

2.2. Initial fibroblast cultures

Approximately 1×10^6 murine fibroblasts (CCL-92) 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, at room temperature in the Cobalto-60 irradiator (type Gammacell), at the CTR/IPEN nuclear irradiation plant.

2.3. Experiments preparation

The experiments with murine fibroblasts as feeder-layer where realized four different times, using 24 well culture plates (1,96 cm²/well area). The cell density was 6×10^4 cells/well. The cells where monitored daily under an optic microscope. These cells were detached from the flask and after coloration with a Tripan Blue solution (0,4%) were counted in a hemocytometric chamber.

2.3.1. Survival curve

In this experiment it was used 120 wells containing irradiated fibroblasts at 60 Gy. Half of the wells of each plate was kept with culture medium D10 and the other half kept with culture medium K- (after 2 days changed for K+). Daily during the first five days and subsequently every 2 days, the fibroblasts were trypsinized and counted. This experiment lasted 30 days.

2.3.2. Comparison between culture mediums used to seed

32 wells containing fibroblasts irradiated with a dose of 60Gy were used for this experiment. Cells in 16 wells were seed with medium culture D10 and after one day exchange with medium culture K- (after 2 days changed for K+). Cells in the additional wells were seed and cultures maintained with medium culture K-/K+. The experiment took 10 days.

2.3.3. Different irradiation doses

Fibroblasts were irradiated at different doses, i.e.: 60, 70 and 100 Grays and seeded in 5 wells per dose. Non-irradiated cells were used as controls. At 24 intervals, representative cultures of each irradiation dose were interrupted, the cells detached chemically by trypsin (0,05%)/EDTA (0,02%) solution and after Trypan Blue coloration, counted and the cell viability noted. This experiment lasted 5 days.

2.3.4. Fibroblasts storage after irradiation

All fibroblasts used in this experiment were irradiated with 60 Gy. In the first 5 wells they were seeded immediately after being irradiated. The other cells were stored in the

refrigerator at (4°C) and later, after one and two days respectively, they were then seeded. Cells were counted every day. This experiment lasted 5 days.

3. RESULTS AND DISCUSSION

In the experiment of survival curve a higher density of cells could be appraised in the cultures when the D10 medium culture was employed, resulting in a less amount of spaces without cells in the fibroblasts layer considering the same time of cellular culture.

Figure 1 shows a visual difference of the cellular layer when medium culture D10 and K were used, respectively, visualized in microscope of inverted light, between days 12 and 13. In the figure 1A close cells can be visualized, the majority with the same format and size. The figure 1B, show the cells cultured with medium K-/K+, which are present with irregular volumes and less as noted by void spaces between actual cells.

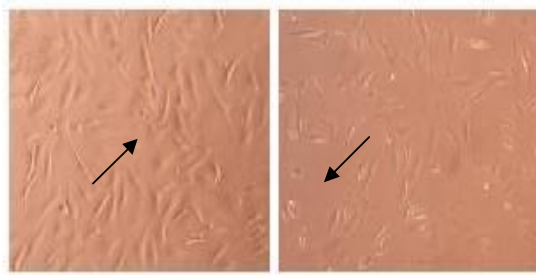


Figure 1. (A) medium D10, cell layer integral (↗); (B) medium K, layer with spaces without cells (↘)

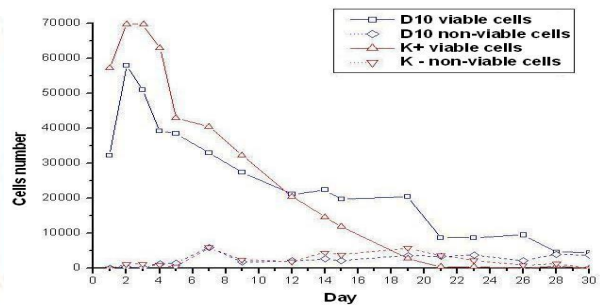


Figure 2. Graphic of fibroblasts counts cultivated with different medium cultures

These results were confirmed by the less the number of viable cells present in cultures with Medium K as per Graph in Figure 2. The number of viable and non-viable fibroblasts, at thirty days of the experiment; express a corresponding survival curve. The low and constant numbers of counted non-viable cells in all wells, kept with reflected the removal of detached cells during the medium changes and chemical detachment of cells.

In Figure 2, a constant decrease in viable cell numbers can be appreciated in both situations; however, the decrease in cell numbers was more evident in the cultures nourished with culture medium K at the final days of the experiment. In the initial stages, higher numbers of viable cells were accounted for in the wells were the medium culture K was used; this situation changes from the 12th day, and remains until the end of the experiment, when higher numbers of viable cells can be obtained from wells nourished with D10 media. This

finding could be explained by the fact that the culture K media contains adenine, a known inhibitor of fibroblasts. Despite, feeder layer kept in medium culture K to have demonstrated inferior quality to the kept one in medium D10 culture, this kept integral and with the necessary characteristics for the culture of the keratinocytes.

Being that the reality of the use of feeder layer, for the culture of keratinocytes, will be its maintenance in medium culture K, a possibility would be to seed these cells in D10 and later substituting the medium K. In this direction the growth and maintenance of the irradiated fibroblasts was followed, during ten days, after to be seed in both mediums. The data in Table 1 indicates that the fibroblasts plated within medium culture D10 had initially a higher number of duplications, with 80% of viable cells present along the observed 10 days of culture. Fibroblasts plated within medium culture K presented a smaller number of initial duplications (maybe as a result of the action of adenine in the medium); however, with the maintenance approximately 90% of the cells remained viable cells within this period of time.

Table 1. Comparison between culture mediums used to seed

Medium type	Viable cells (average) a long of time in days							
	0°	1st	2nd	3rd	4th	6th	8th	10th
medium D10	59,875	58,500	99,000	98,875	83,250	82,500	80,500	78,750
medium K	56,375	55,750	63,125	66,500	62,500	60,750	59,250	59,750

Table 2. Impact of irradiation doses upon viability

Irradiation Dose (Gy)	Viable cells (average) in time (days)					
	0	1st	2nd	3rd	4th	5th
0	56,000	116,750	242,000	547,500	1,120,000	2,210,000
60	66,500	64,750	98,875	94,750	83,625	82,750
70	53,375	39,750	39,750	33,750	30,500	31,750
100	56,875	32,250	26,750	26,375	22,750	21,125

The values indicated in Table 2, demonstrate that the different doses of radiation applied to the cells influenced fibroblast viability in culture. The non-irradiated fibroblasts proliferated and cell numbers practically doubled in number each day. The best results as far as viable cell numbers were found in the dose level of 60 Gy for this cell line. The cells still duplicated in the first day, probability because they are at different stages of duplication, and in the second day had ahead kept the number of cells forming a compatible layer to be used as feeder layer. The cells irradiated with 70 and 100 Gy had quickly been modified, remaining 60 and 40% of the amount of seed cells respectively. With these results, we confirm that the radiation dose needs to be enough to stop the duplication cells but not higher in order to avoid cell damage.

Morphologically, the more high a dose used in the irradiation of these cells, worse the characteristic of feeder layer as shows Figure 3.



Still observing the Figure 3 it is possible to see that the quantity of the cells that did not receive any radiation dose or that received 60 Gy radiation adhered better to the plate surface. It is also possible to observe larger voids between cells denoting less cell quantity after five days in the 70 and 100 Gy range of radiation dose, reflecting the deleterious effect of the higher doses of irradiation. In Table 3 the numbers of fibroblasts plated immediately, at 24 h and at 48 h following irradiation.

Table 3. Fibroblasts storage after irradiation

Time of storage	Viable cell (avarage) a long of time in days					
	0	1st	2nd	3rd	4th	5th
immediate use	55,500	49,000	71,000	70,500	73,500	65,500
1 day	55,500	49,500	69,000	69,500	68,000	65,250
2 day	48,500	53,500	71,000	72,500	72,000	69,250

Analyzing the results, we could verify that the fibroblasts remained viable for plating even after two days of irradiation if stored under refrigeration at 4°C; also, that they could be maintained in culture as a potential feeder layer for at least five days after been plated. These cells presented with a similar behavior to cells seeded immediately after irradiation.

3. CONCLUSIONS

CCL-92 fibroblasts formed adequate feeder layers for co-cultures when both culture medium was used. Layer kept the necessary characteristics for co-cultures in both types of culture medium. Different doses of radiation influence in the quality of feeder layer and it is possible to store, under refrigeration, the irradiated fibroblasts, at least during two days, before uses them as feeder layer.

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