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Original Article

Reporting the Effect of Cell Seeding Density on Growth Pattern of Cancer Cell Lines

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Abstract

In vitro studies on cancer cell lines still constitute a major part of cancer research. It is very important that the researchers consider the importance of basic conditions of working with different cell lines. To the best of authors' knowledge, this study is the first one to report the effect of seeding density on the growth curve of three human cancer cell lines. These cell lines include human breast adenocarcinoma MCF-7 cell line, human ovary carcinoma A2780 cell line and human melanoma A375 cell line. The cell lines were seeded at 4 different seeding densities: 4000, 5000, 15000 and 50000 cells/cm^2 of culture surface. Following seeding the cells and on daily basis, cell count was done with trypan blue assay. The experiment was done in triplicate. The growth curves of the cells were constructed using Microsoft Excel and population doubling time (PDT), lag time (LT) and saturation density (SD) were calculated. The results showed that the seeding density affects the general pattern of the growth curve. At lower seeding densities, the growth curves have a more expected pattern rather than in higher seeding densities. Also, seeding density does not affect the LT, while it has some effects on SD and PDT.

Keywords: A2780, A375, Cancer, Growth Curve, MCF-7, Seeding Density.

1. Introduction

A big part of cancer research, defining the mechanisms of cancer progression, treatment, recurrence and so on is nowadays relying on in vitro studies. *In vitro* studies are based on cancer cell culture. At this stage, what need to be carefully taken care of are the conditions at which the cells are grown at. These conditions have great impact on the final results of the study and include optimum conditions for freezing, thawing, freezing frequency, incubation situations, using the optimum media, etc [1-4]. Many scientists have studied the effects of different environmental conditions on cells growth patterns. For example, the effect of alkaline pH on the

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growth of mammalian cells were studied by Burroni and Ceccarini [5] and Zetterberg and Engström [6].

Cancer cell lines have their specific growth pattern [7, 8] and this growth curve defines specific characteristics of that cell line [9, 10]. These characteristics include population doubling time (PDT), lag time (LT) and saturation density (SD). PDT is defined as the time required for the cell population to double in count. LT is the time required for the cells to start the exponential phase of growth and SD is the maximum cell count that the cell line reaches at plateau state.

It is expected that before any *in vitro* culture study, such cell specific characteristics be evaluated. As a common procedure, *in vitro* cancer studies are done at exponential phase of cells' growth curve. However, many researchers do not consider the effect of seeding density on the growth pattern of the cells. In the current study, we have evaluated the effect of seeding density on the growth pattern and measured growth curve related characteristics of different human cancer cell lines.

2. Material and Methods

2.1. Materials

Two of the cell lines, including MCF-7, human breast adenocarcinoma, and A375, human skin melanoma were purchased from by Iranian biological resource center. Human ovary carcinoma A2780 was prepared from the National Cell Bank of Iran. Cell culture media (RPMI-1640), fetal bovine serum and penicillin/streptomycin were purchased from Gibco® BRL. All other materials used in the study were purchased form Sigma®.

2.2. Methods

2.2.1. Cell Culture Methods

Cell lines were thawed and passages for at least three passages [11, 12]. The cells were then seeded at three different densities of 4000, 5000, 15000 and 50000 cells/cm^2. At 24 hour intervals, cell count was performed as described below.

2.2.2. Cell Count

Cell count was performed based on trypan blue assay as previously described [7, 10-13]. Briefly, the cells were trypsinized, mixed with trypan blue with the ratio of 1:1 and 20 µL of this mixture was placed on a hemocytometer. The number of live cells was counter under an inverted microscope.

2.2.3. Statistical Analysis

Growth curves of each cell line and related calculations were performed using Microsoft Excel® software, 2013. Cells' doubling time (hour), lag phase (hour) and saturation densities (cell/cm^2) were calculated based on the explanations by Freshney *et al.* [14, 15]. Comparisons were analyzed in Graphpad PRISM® software, version 5. *P* value<0.05 was considered as the level of significant difference.

3. Results and Discussion

There are many factors that affect the growth pattern of cancer cells *in vitro* and influence the final results of any study. Some of them are very well-known and some are sometimes neglected. In the current study, for the first time, we have evaluated the effect of seeding density on cancer cells' growth curve pattern. Hence, 3 different cancer cells were grown at 4 different seeding densities; 4000, 5000, 15000 and 50000 cells/cm^2. The final growth curves of these cells are shown in Figure 1.

In MCF-7 cell line, cells seeded at the density of 4000/cm^2, showed the longest plateau phase. The growth curve at this seeding density is closest to the expected growth curve rather than the other MCF-7 growth curves. As it is observed in this figure, increasing the seeding density decreases the time cells spend at plateau phase. Interestingly, LT for the cells to start the exponential phase is the same. Although, based on the data shown on Table 1, there are some differences between the calculated LT at these 4 different seeding densities, statistics analysis could not detect any significant differences between these values (p<0.05). The saturation density is somehow higher in higher seeding densities (5000, 15000 and 50000 cell/cm^2) (Table 1).

There is a different story for A375 cell line. In this cell line, like MCF-7, the longest plateau phase belongs to the lowest seeding density (Figure 1). The plateau phase in curves with the starting points of 5000, 15000 and 50000 cells/cm^2 decreases, respectively. However, unlike MCF-7, the shortest LT belongs to the curve with 4000 cells/cm^2. The longest lag phase belongs to the curves with the starting points at 5000 and 15000 cells/cm^2. Regarding SD, like what was observed for MCF-7, the curve with 4000 cells/cm^2 has the lowest SD amongst the 4 curves.

Human ovary carcinoma cell line, A2780, shows the same pattern for plateau phase. The results show that the longest plateau phase belongs to the curve with 4000 cells/cm^2 starting seeding density. SD of this cell line is also the least at this cell seeding density and it slightly increases as the seeding density increases (Table 1). However, LT is the same for all A2780 growth curves.

In the current study, we seeded the cells at different densities and observed that at higher cell densities, the cells enter the death phase of the growth curve much faster. This was also accompanied by faster change in media pH, as observed by media colour. In other words, at higher cell densities, the colour of the media became yellowish much faster than the cells

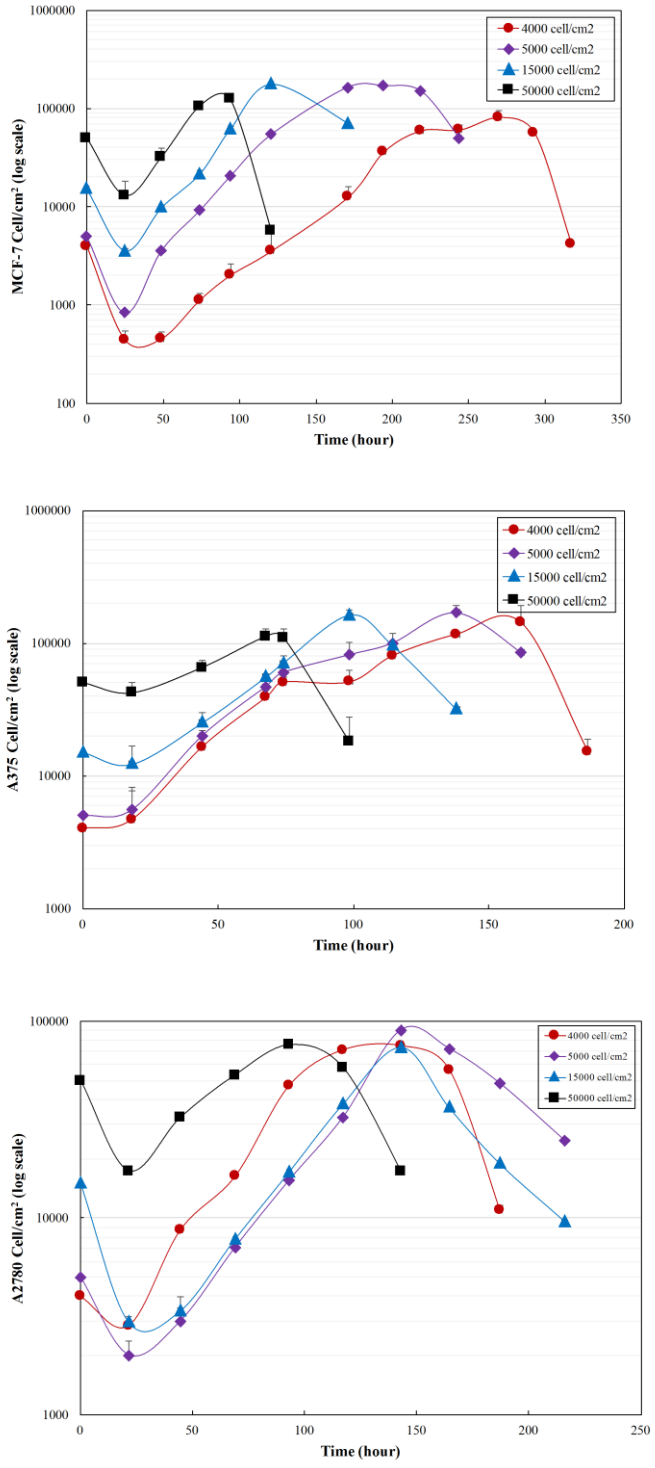

Figure 1. Growth curve of MCF-7, A2780 and A549 cell lines, following seeding at 3 different seeding densities; 4000, 5000, 15000 and 50000 cells/cm^2. Data are presented as mean ± standard deviation.

seeded at lower densities. Although, it is said that cancer cells, compared to normal cells, can grow in a broad range of pH specially in acidic pH [16], it wasn't the case for the studied cell lines. Yet, a proper pH

Table 1. Saturation density (SD), lag time (LT) and population doubling time (PDT) of MCF-7, A375, and A2780 cell lines after seeding at 4 different seeding densities; 4000, 5000, 15000 and 50000 cell/cm^2.

Cell lines	Characteristic	Primary cell seeding density/cm^2			
		4000	**5000**	**15000**	**50000**
MCF-7	SD[1]	80000	200000	200000	200000
	LT[2]	15	20	20	20
	PDT[3]	14	10	16	14
A375	SD	50000	170000	170000	150000
	LT	15	17	17	17
	PDT	14	14	23	38
A2780	SD	71000	90000	361000	721000
	LT	20	20	20	20
	PDT	15	15	15	15

[1] Saturation Density (cell/cm^2)
[2] Lag Time (h)
[3] Population Doubling Time (h)

measurement is required to discuss this point of view.

The results also show that the cells at higher seeding densities have a very short plateau phase and reach the death phase faster. Seeding density affects the SD in most cases and has a minor effect on LT.

Unlike what Ceccarini and Eagle reported [16], these cells which were cultured at higher densities, detached cell layers were not observable in the supernatant media. We already are aware of the growth inhibitory effect of normal cells in interaction with one another. This inhibitory effect is known as contact inhibition of proliferation [16]. However, contact inhibition is a phenomenon known for normal cells and fibroblasts and it was believed that cancer cells, due to their invasiveness, might not be obedient to this rule [17-20]. Cancer cells, as Hanahan *et al*. states are masters of their own proliferation as they produce signals to initiate cell proliferation in the nearby cells and they are not affected by contact inhibition of proliferation. [21]. However, contact inhibition properties are shown to be active on some cancer cells, such as malignant melanocytes [22]. As the studied cells enter the death phase following the very short plateau phase, possible contact inhibition in these cell lines is a matter of further studies.

4. Conclusion

The overall conclusion of this study is that the pattern of cell line's growth curve is dependent on the seeding density. The growth curves that are constructed from 4000 cells/cm^2 show a more expected pattern of growth curve, which includes lag phase, exponential phase, plateau phase and death phase. Increasing the seeding density, changes

some characteristics of the cells such as PDT and SD. LP is less affected by seeding density. These changes are cell line specific. It is thus suggested that researchers consider the effect of seeding density before running any experiments.

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