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Research Article

Optimization of Protocol for Maintaining and Freezing Caco-2 Cells

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Abstract

Human Caco-2 cells are used as model of intestine for absorption studies. However standardization of growth conditions is challenging for these cells as it requires a long growth period of around twenty one days for most absorption associated studies. In this study we have standardized various growth associated parameters and procedures related to Caco-2 cell cultures. The Caco-2 protocols are recently developed in our laboratory include, the time required for trypsinization of cell in correlation with the age of subculture, freezing mixture type, quantity of DMSO and seeding density of cells while freezing. The cells are also seeded at low density as compared to present in literature which gave better results for a 21 day old differentiated flask. Since the Caco-2 cell line is integral part of absorption and *in vitro* toxicology tests, standardized protocol inclusive of all the detailed procedures for growing and differentiating Caco-2 cells would help researchers in maintaining and preserving the cells.

Keywords: Caco-2; Trypsinization; Seed density; Freezing; Thawing.

Introduction

Caco-2 model is employed in pre-clinical investigations of drugs and food. FDA recommends Caco-2 model as integral component of biopharmaceutics classification system owing to the fact that Caco-2 expresses cytochrome enzymes, transporters and enterocytes of human small intestine [1, 2]. Caco-2 cells serves a good model to elucidate the roles of the physical and biochemical barriers to drug absorption imposed by the epithelium lining of intestine [3].

In the intestine, a single layer of epithelial cells covers the inner intestinal wall and forms the rate-limiting barrier to the absorption of dissolved drugs, similarly a well differentiated human epithelial cell monolayer *invitro*, allows the prediction of oral drug absorption in humans [4]. The human colon carcinoma cell line Caco-2 has been found to serve this purpose well [5]. In culture, this cell line slowly differentiates into monolayer with a differentiated phenotype with many functions of the small intestinal villus epithelium. We in our laboratory have standardized the protocol for time required for trypsinization of Caco-2, freezing mixture composition and cell density used for freezing cells. Standardization has been done as many researchers find discrepancies in reports between laboratories for the same cell line [6-8]. Since Caco-2 remain exclusive model for toxicological studies, our aim in this study is to standardize, comprehensively practiced protocols in detail and provide additional insight into maintenance and sub-culturing Caco-2 cell line.

Materials and methods

Cell culture

Caco-2 cells were purchased from National Center for Cell Sciences, Pune, India. They were studied within 34–39 passages. Cells were grown to confluency in MEM [HiMedia, Mumbai, India] supplemented with 10% fetal bovine serum [FBS, Gibco, Thermo Fisher, USA], 4mM glutamine, 1% non-essential amino acids, 100 IU/mL penicillin and 100 ug/mL streptomycin [HiMedia, Mumbai, India]. They were maintained in a humidified 5% CO2 / 95% air atmosphere at 37°C using 25/75 cm^2 tissue culture flasks [Nunc, Nalgene Nunc International, Rochester, NY, USA].

Cell line maintenance and sub-culturing

The cells were sub-cultured once a week, at about 70% confluence, and the medium was

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changed three times a week. When the cell line reached a density of 70% confluence, medium was removed completely and the cells were washed with 2ml PBS. Cells were incubated with 2ml 0.25% trypsin-EDTA solution [Gibco, Thermo Fisher, USA] for 4 mins at 37°C, 5% CO_2. The cells were resuspended in 5ml of complete medium to neutralize the action of trypsin. After which the cells were centrifuged at 1600 rpm for 5 min. The pellet was re-suspended in 1ml of complete medium. The cells counted and were seeded with a cell density of 3 × 10^3 cells/ cm^2. The media was changed every 2 days [9].

Time for trypsinization

In order to determine the optimum time of trypsinization in correlation with the day of subculture of cells, the T-25 flasks were trypsinised from 6^{th} day of subculture till 22^{nd} day for time ranging from 1.5 min to 8 min. The cell density was calculated after trypsinization on each of the above mentioned days. A graph was plotted for time required for trypsinization versus the days of subculture. For all the experiments 2ml of Trypsin-EDTA [0.25%] was used and incubation was carried out at 37°C, 5% CO_2.

Standardization of cell freezing mixture

Caco-2 cells were harvested by trypsinisation using the procedure described in the subculturing section. After the centrifugation step and removal of the medium, cells were re-suspended in 2 mL of FBS, counted. Cells were distributed in polypropylene cryovials [Nalgene Nunc International] to obtain final cell density of $2*10^6$ cells/ml and freshly prepared, ice-cold freezing mixture was added. Cells were frozen using Mr Frosty [Nunc, Nalgene Nunc International, Rochester, NY, USA] at -80°C for 24 h followed by transfer in liquid nitrogen cans. Different freezing mixtures used were: Mix A contained 90% FBS + 10% DMSO, Mix B contained 95% FBS + 5 % DMSO, Mix contained 45% FBS+45%MEM + 10% DMSO and Mix D contained 50% FBS+45%MEM + 5 % DMSO. Cryo vials were kept at -80^0 C for one day and transferred to liquid nitrogen tank. The cells were thawed 30 days post freezing.

Standardization of cell seeding density for freezing

After standardizing the freezing mixture for Caco-2 as 90% FBS with cells + 10% DMSO the seeding density for freezing was standardized. Cells were frozen with a seeding density of 0.5, 1, 1.5, 2, and 2.5 x 10^6 cells/ml. For cryopreservation , the freezing mixture with cells were saved in cryovials and frozen using Mr Frosty [Nunc, Nalgene Nunc International, Rochester, NY, USA] at -80°C for 24 h followed by transfer in liquid nitrogen cans. The cells were thawed, counted using trypan blue for viability after 30, 60 and 90 days post freezing.

Result and discussion

Time for trypsinization

Human intestinal Caco-2, as mentioned in the literature differentiates spontaneously in culture and has been used as a model for intestinal absorption studies. Differentiated Caco-2 cells exhibit a better morphological and functional enterocytic differentiation than does other colon carcinoma cell-lines [10,11]. It grows synchronously forming a homogenized and polarized monolayer. Through this study, effort has been made to standardize time required for trypsinization, freezing mixture components and seeding density for freezing Caco2 cells. Trypsinization is an important and integral step in cell culture. However the exposure of Caco-2 cells to trypsin should be as short as possible as this process affects cell viability [12]. Several researchers have reported the incubation time starting from 4 minutes to 15 minutes [13]. This time duration is dependent on several parameters such as concentration of trypsin, passage number of cells, confluence etc. This leads to variability in determining the exact time and hence further leads to over exposure to the enzyme. However from current study we state that it's more dependent on subculture time. To investigate the influence of subculture day on action of trypsin, the flasks were subjected to trypsinization from second day till twenty second day of subculture. Time required to achieve maximum disassociation of confluent cells in a monolayer increased with day of subculture half minute for two day old flask to eight minutes for twenty two day old flask (Figure 1). Trypsinization is the prime step in cell culture experiments and also in routine maintenance of cells. Most of the protocols in literature state an empirical time period of trypsinization. Since excess trypsinization leads to cell clumping and affects cell viability, accurate measurement of time is

imperative. From our findings, the time required for trypsinization is related to day of subculture.

Standardization of cell freezing mixture

The important step is washing the monolayer with PBS or Hanks Balanced Salt Solution, at least twice to remove traces of residual serum, prior addition of trypsin. The freezing mixture of 10% DMSO in heat inactivated FBS yielded better results than the other three freezing mixtures (Figure 2).

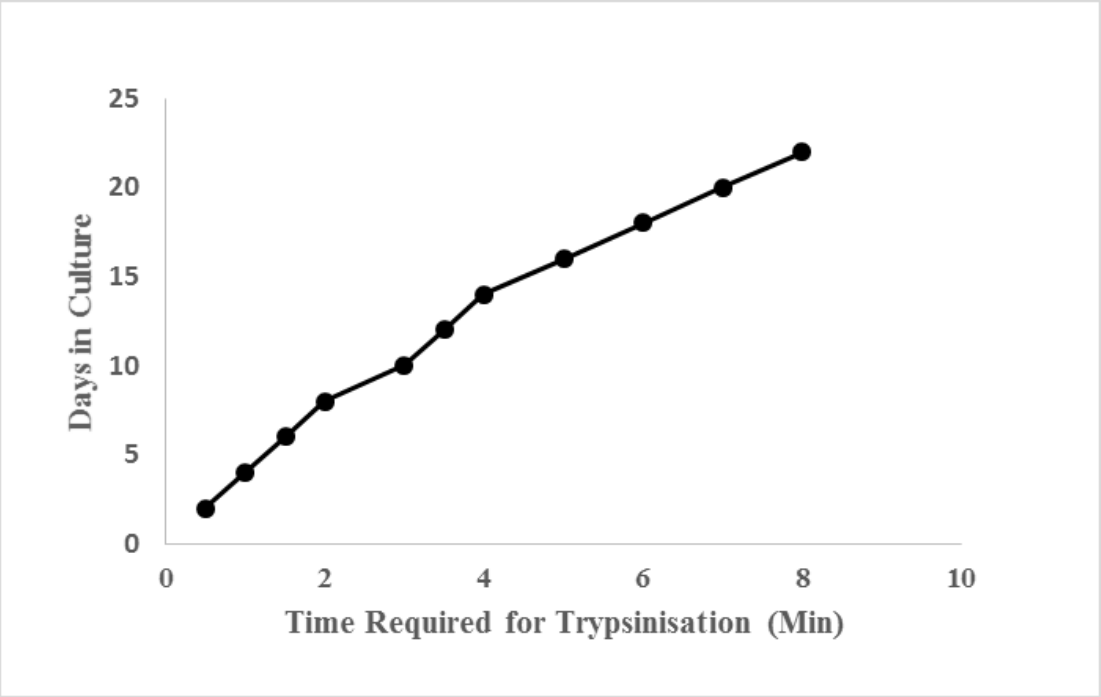

Figure 1. Effect of subculture time on trypsinization

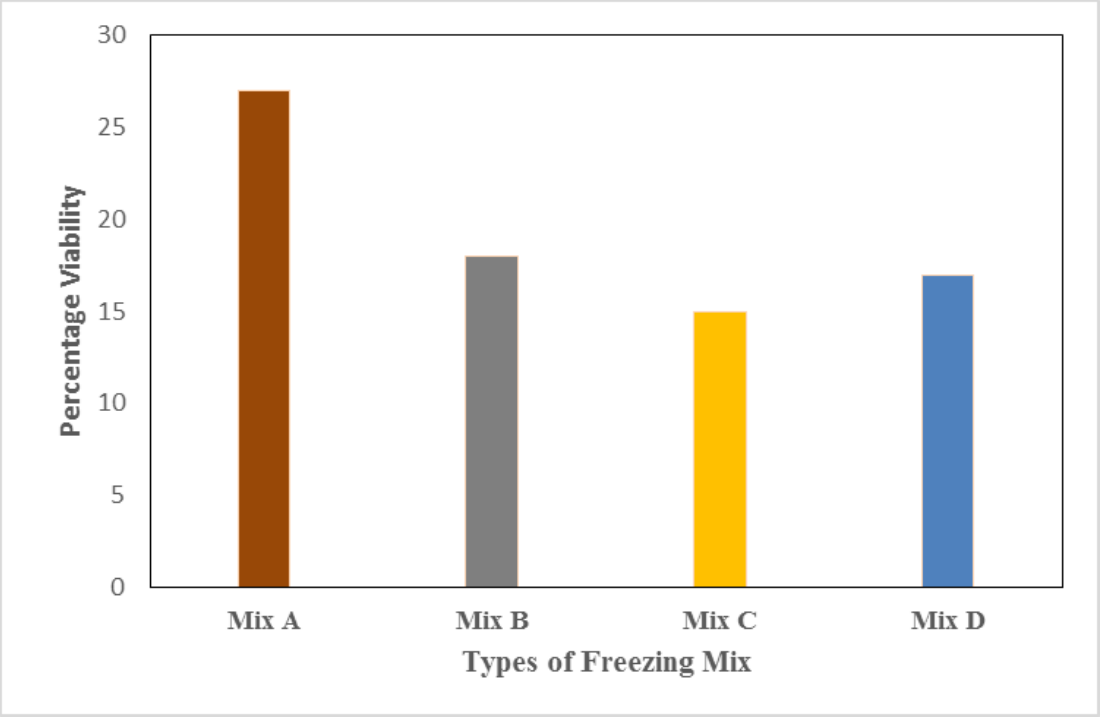

Figure 2. Effect of Freezing mix on viability of cells post thawing, Mix A: 90% FBS + 10% DMSO, Mix B: 95% FBS + 5 % DMSO, Mix C: 45% FBS+45%MEM + 10% DMSO. Mix D: 50% FBS+45%MEM +5 % DMSO

So for all the experimental conditions to achieve maximum viability with different seeding densities 10% DMSO in FBS was preferred. An optimized freezing protocol

should guarantee adequate cell viability. We evaluated the impact of four cryo-protectant combinations on cell viability upon thawing. Although earlier research suggests the use of complete medium with 10% FBS during freezing, our findings indicates that 90% FBS supplemented with 10% DMSO was best for freezing Caco-2 cells, followed by 95% FBS and 5% DMSO [14].

Standardization of cell seeding density for freezing

Viability study was done over a period of three months with five different cell density namely 0.5, 1, 1.5, 2, and 2.5 x 10^6 cells/ml. This was done to assess the impact of freezing conditions on the cell count post thawing. Upon thawing, post thirty days freezing, 2.5 x 10^6 cells/ml, the highest cell density vial, could retain 27.6% viability followed by 2 x 10^6 cells/ml vial which retained 27%. Lower cell densities of 0.5, 1 and 1.5 could retain 20.6, 20 and 19%. This indicated that cell densitiy of 2 x 10^6 cells/ml is adequate and any higher cell density than this does not give an additional edge. Also all cell densities lower than 2 x 10^6 cells/ml, retain similar viability post thawing but on a lower side. Post sixty days there was reduction in viable count highest being 22% and lowest being 10.4%. Similar results were obtained for viable counts on 90th day post thawing. The counts were significantly reduced for initial lower seed densities [0.5, 1, 1.5 x 10^6 cells/ml] than for higher seed densities [2, and 2.5 x 10^6 cells/ml]. Higher seed densities could retain 20 and 19% viability (Figure 3).

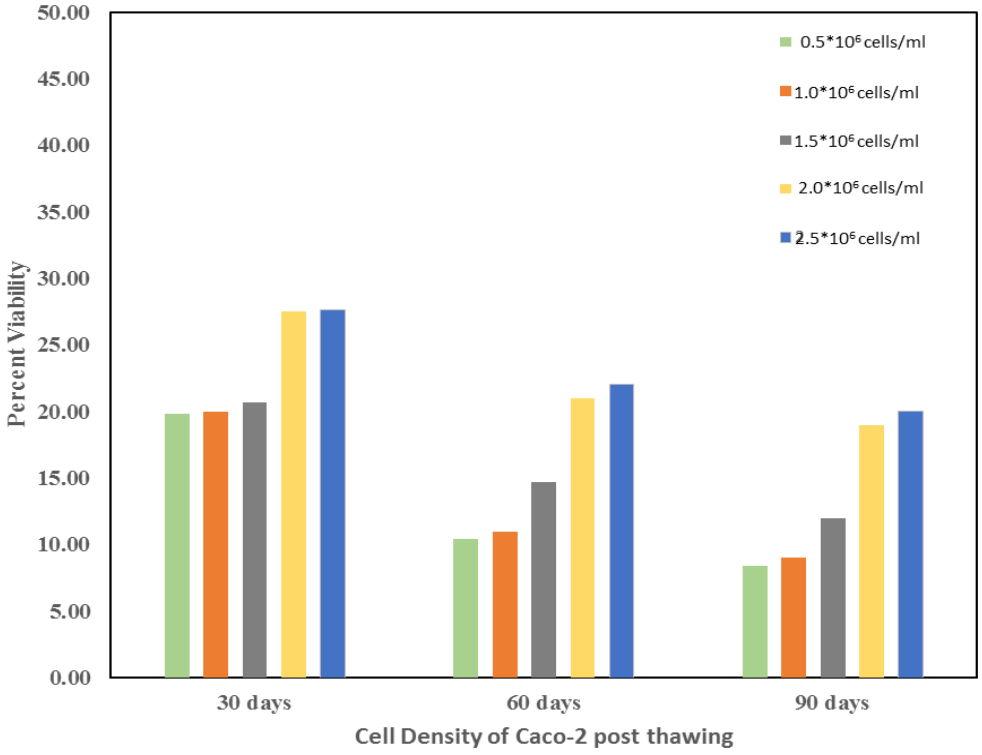

Figure 3. Effect of cell seed density on viability of Caco-2 cells post thawing

Hence these cell densities are most suited for freezing procedures related to Caco-2 cells. This could be due to use of manual method of freezing instead of automated temperature controlled freezers. The Caco-2 cell line, established by Fogh and co-workers in 1977, is most widely used human colon adenocarcinoma for the screening of cytotoxic effects of anti-tumor drugs and for the study of drug resistance mechanisms [13]. Seeding density of Caco-2 cells during freezing is also a crucial parameter. Cell density of 2*10^6 cells/ml was found to be optimum for freezing. Lower seeding density for cells decreases the cell count upon revival.

Conclusions

There is paucity of standardized basic protocols for freezing and routine maintenance of Caco-2 cells in existing literature. Hence due to lack of standardization of experimental designs and protocols, it is tedious to maintain Caco-2 cells for a long time duration for experimental work.

Hence this results in difficulty in drawing conclusions on investigations. Hence this study focuses on standardization of basic yet useful protocols for maintaining Caco-2 cells. However, it should be considered that this study, reports about short-time [up to 90 days] storage and further investigations are necessary for long-time preservation.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

Acknowledgment

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