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Metabolic Analysis of Fibroblast Conditioned Media and Comparison with Theoretical Modeling

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Abstract**—**Understanding the consumption and production of various metabolites of fibroblast conditioned media is needed for its proper and optimized use in expansion of pluripotent stem cells. For this purpose, we have used the HPLC method to analyse the consumption of glucose and the production of lactate over time by mouse embryonic fibroblasts. The experimental data have also been compared with mathematical model fits. 0.025 moles of lactate was produced after 72 hrs while the glucose concentration decreased from 0.017 moles to 0.011 moles. The mathematical model was able to predict the trends of glucose consumption and lactate production.

Keywords**—**Conditioned media, HPLC, metabolite analysis, mouse embryonic fibroblast.

I. Introduction

PLURIPOTENT stem cells, by virtue of their properties of unlimited self renewal and capacity to differentiate into multiple lineages, are important for various scientific purposes like tissue engineering, regenerative medicine, *in vitro* models for complex diseases, etc. Traditionally, these cells are expanded as co-cultures on a feeder layer of mouse embryonic fibroblasts (MEF). With time, various other fibroblast lines have also been tested and used. For example, human fibroblasts have been used as a 'feeder layer' for human pluripotent stem cells [1]. In the absence of a feeder layer, pluripotent stem cells rapidly and randomly differentiate, highlighting the importance of the presence of the feeder cells as matrices. However, concerns regarding cross species contamination and lack of purity in pluripotent cells due to the presence of feeder cells have led to the use of fibroblast conditioned media, specialized commercial media and/or specific growth factors for the expansion of pluripotent cells in absence of a feeder layer [2-6].

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The fibroblast conditioned media is hypothesized to contain a plethora of unknown growth factors and cytokines secreted by the feeder cells that are needed to culture the pluripotent stem cells *in vitro*. Efforts have been made to identify and classify the different factors secreted by the fibroblast cells with the ultimate goal of using a completely defined and optimized conditioned media or manufacture a robust synthetic media for expansion of pluripotent stem cells. Prowse et al. [7] demonstrated that conditioned media derived from mouse and human fibroblasts contain a complex mixture of growth factors, extra cellular matrix proteins and various differentiation factors involved in the growth of human embryonic stem cells. They also showed that the different fibroblast lines had only slight differences in the number and type of proteins secreted. Although a large number of the feeder cells' secreted proteins are identified now, many still remain to be identified, classified and purified. Hence, the use of conditioned media is still preferred in many laboratories to date. While all these experiments have identified various protein components of the conditioned media, very little is known about the metabolites present. This information is extremely crucial for a truly optimized conditioned media, especially the knowledge of lactate accumulation.

Accumulation of lactate/lactic acid has been reported to be one of the potential growth inhibitors of animal cells as early as 1958 [8]. It is produced mainly due to the metabolism of glucose but is reported to be produced in a small amount from glutamine also [9]. Lactic acid had been showed to have a significant effect on cell proliferation, metabolism and differentiation. It has been reported that various cell lines have differing tolerance for lactate accumulation. For example, BHK cells have a higher lactate tolerance than a hybridoma cell line [10]. It was reported that proliferation of hematopoetic stem cells ceased at a lactate concentration of 20 mM and above [11]. Embryonic stem cells were reported to be highly sensitive to lactate accumulation. Ouyang et al. [12] demonstrated that a lactic acid concentration of 1.5gm/litre (around 0.016 moles) was highly detrimental for the ESD3 mouse embryonic stem cell line [12]. In practice, conditioned medium is mixed with fresh medium to nullify the detrimental effects.

Metabolic profiling of various animal cell lines like CHO, BHK, hybridoma, and mesenchymal stem cell has been carried out to date using enzymatic assays or biochemical analyzers. Ouyang et al. [12] and MacIntyre et al. [13] published an approximate range of lactate concentration for

mouse and human fibroblast conditioned media which had been used for their experiments. However, the collection procedure and time are different in both these studies. Knowledge of the metabolic profile of the feeder cells would be helpful in deciding not only the conditioned media collection period but also the ratio of diluting conditioned media with fresh media if and when needed.

In this work, we present the first experimental metabolic profile for mouse embryonic fibroblasts; this being one of the most commonly used feeder cell line using the simple and easily available analytical process of High Pressure Liquid Chromatography (HPLC). The mathematical model presented here fits the experimental data very well allowing the users to extrapolate the data beyond the experimental time line if needed.

II. Materials and Methods

A. Cell Culture and Preparation of Conditioned Media

Mouse embryonic fibroblasts (MEF) (SNL cell line procured from the lab of Prof. Allan Bradley, Director Emeritus, Welcome Trust Sanger Institute, United Kingdom) were cultured in standard MEF medium comprising Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, Life Technologies Corporation,) supplemented with 10% Australian origin fetal bovine serum (JRH Biosciences, SIGMA), non essential amino acid (Invitrogen, Life Technoloiges Corporation), GlutaMAX^{TM}(Invitrogen) and antibiotic- antimycotic solution (Himedia, India) on T25 tissue culture flasks. On reaching 70- 80% confluency, the cells were trypsinized and plated on to 24 well plate at a seeding density of approximately 50,000 cells/cm^2 and incubated overnight in a 5% CO_2 concentration incubator at 37°C. After incubation, the cells were washed twice with PBS to remove any non adherent cells. 1 ml of fresh MEF media was added to each well (~0.5ml/cm^2) and incubated. The time point for fresh media addition was considered as 0 hr. Conditioned medium was collected at regular intervals, centrifuged at 3000xg for 10 minutes to precipitate out any cell debris. The supernatant was collected for metabolite analysis. Cell count was done using hemocytometer in triplicates for each time point.

B. Metabolite Analysis

Estimation of glucose and lactate concentration in conditioned media was done using High Pressure Liquid Chromatography (HPLC). The system is an indigenous system with an Aminex HPX- 87H cation exchange column (Biorad). The mobile phase used was 5mM H_2SO_4 at a flow rate of 0.6ml/min and was operated at an elevated temperature of 60°C. Monitoring and detection of glucose was done using a refractive index (RI) detector and for lactate, a UV detector was used operating at 214nm.

Standard Curve: Standard curves for glucose and lactate were prepared by analysis of serially diluted solutions of pure glucose and pure lactic acid (Merck, Millipore,). The ability of the instrument to separate and detect glucose and lactate was tested using mixture of pure glucose and lactic acid and also using mixture of standard media and lactic acid (data not shown here).

C. Mathematical Modeling

For the purpose of mathematical modeling, the 24 well plate is considered to be a batch system wherein the cells grow on the present metabolites. The principle of the model used is based on that of Higuera et al. (2009). Several other publications have also considered metabolite modeling of animal cells in culture. The system can be represented by a set of mass balance equations (1) to (4). The rate of change of viable biomass (total cell number in this case) is given by the equation

$$\frac{dX_v}{dt} = X_v.(\mu - \mu_d) \quad (1)$$

This equation includes both viable cell growth ($X_v.\mu$) and cell death ($X_v.\mu_d$).

$$\mu = \mu_{max}.\frac{X_g}{K_g + X_g} \quad (2)$$

Equation (2) gives the specific growth rate of the cells (μ) . It is based on Monod type kinetics for glucose.

The rate of change of the amount of dead cells (X_d) is represented by the equation

$$\frac{dX_d}{dt} = X_v.\mu_d \quad (3)$$

The rate of change of the mass balance of metabolites (X_m) is given by the following equation:

$$\frac{dX_m}{dt} = \pm q_m.X_v - k_{md}.X_m \quad (4)$$

Here, the subscript *m* represents metabolites (glucose or lactate). $q_m$denotes the specific metabolite reaction rate per cell per hour. q_m is positive or negative depending on whether the metabolite is produced or consumed respectively. k_{md} represents the metabolite degradation rate constant.

Doubling time for cells was estimated using the equation

$$t_d = (t_2 - t_1) \times \{\frac{\log(2)}{\log(q_{2/q_1})}\} \quad (5)$$

Here,q_1 and q_2 represent total cell number at times t_1 and t_2 respectively. Maximum specific growth rate was calculated indirectly from the doubling time.

The equations (1) to (4) represent a set of ordinary differential equations (ODEs) which were solved using differential equation solver ODE 45 in Matlab (MatlabR2007a). Initial values of cell density, glucose and lactate concentrations, specific metabolite reaction rates for glucose and lactate, and maximum specific growth rate were obtained for experimental data. The value of the Monod

constant was taken from literature. Two different values of the Monod constant were tested for comparison purpose.

The following assumptions were made for experimental purposes and for solving the differential equations:

a. Visual assessment using trypan blue showed very few dead cells. Hence, cell death was taken to be negligible and μ_d was assumed to be 0.
b. As per the literature, degradation of glucose and lactate is negligible. So it was assumed that k_{md}= 0 for glucose and lactate metabolic profiling.
c. The cell population was assumed to be a homogenous one, i.e., a single cell can represent the whole population. Also, it was assumed that the cells were not subjected to random variability.

III. Results and Observations

The present study focuses on the growth and metabolic profiles of mouse embryonic fibroblast (SNL cell line). Viable cell numbers, glucose concentrations and lactate concentrations were measured experimentally and also compared with a simple theoretical model. The cells used here were within the passage numbers 43 to 48.Based on the change in media colour, visible pH change was observed after 48 hrs. This knowledge is expected to help in proper collection and use of MEF conditioned media for the growth of pluripotent stem cells. The detailed results are discussed below.

A. Cell Growth

In a typical batch reactor, the cell number varies with time: an initial lag phase where there is slow cell growth; and an exponential growth follows that is characterized by a rapid increase in cell number. As observed from the experimental data and the mathematical model here, the lag phase for the mouse embryonic fibroblast cells in this case is very small. An average cell viability of around 70% or more was seen at time point 0 hrs after overnight attachment of the SNL fibroblasts. Fig. 1 shows the viable cell number counted experimentally and predicted by the mathematical model for two different values of K_g (Monod constant) over a time period of 72 hrs. The values tested were 0.4 mM [14-16] and 0.6 mM [17].It was observed from the mathematical model that changing the value of K_g did not have any visible effect on the viable cell number.

Fig. 2 shows that there was a four-fold increase in viable cell number over 72 hrs. A visual inspection following trypan blue staining showed very few dead cells and hence cell death was assumed to be negligible. To corroborate this observation, hypothetical values of cell death constant were used for the mathematical model. Fig. 3 shows that the theoretical curve with μ_d = 0 is indeed the best fit for the experimentally obtained data, thus supporting our assumption.

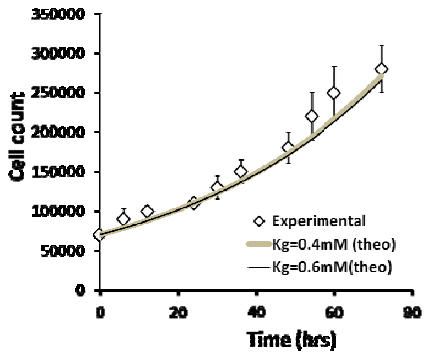

Fig.1 Comparison of experimental and theoretical viable cell count at different values of Monod Constant. Experimental values plotted are Mean±SEM (n=4)

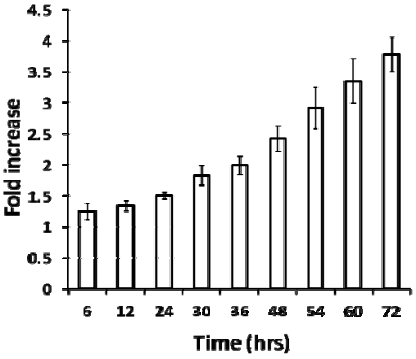

Fig. 2 Fold increase in cell number over time. Data represents Mean±SEM (n=4)

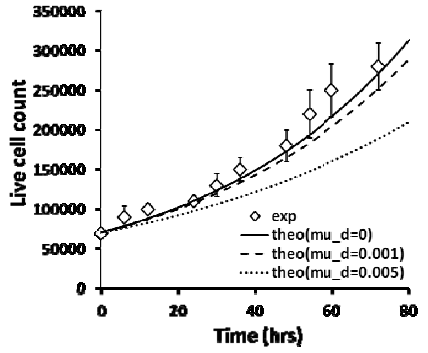

Fig.3 Comparison of theoretical viable cell count at different μ_d values with experimental data. Data represents Mean±SEM (n=4)

B. Glucose and Lactate Metabolism

Glucose and lactate concentrations were measured using the HPLC method. Experimentally, it was observed that glucose concentration had depleted to 0.011 moles (around 2.03mg/ml) from 0.017 moles after 72 hours. The concentration of lactate produced reached a value of around 0.025 moles (approx. 2.23mg/ml) in the same time period. The initial value of lactate was $1.6x10^{-4}$ moles; this may have been due to the serum present in the media itself.

As done previously, two different values of K_g (0.4 mM and 0.6 mM) were used for the theoretical estimation of glucose and lactate concentrations. Similar to the observation in the case of cell number, the change in the concentrations of glucose and lactate due to the change in the value of K_g was negligible. Fig. 4 and Fig. 5 show profiles of glucose consumption and lactate production for the SNL fibroblast cell line, respectively.

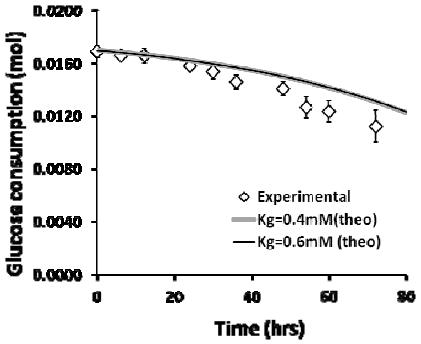

Fig. 4 Comparison of theoretical and experimental values of glucose concentrations at different time points. Data represents Mean±SEM (n=4)

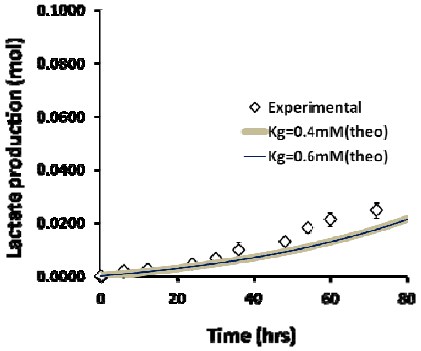

Fig. 5 Comparison of theoretical and experimental values of lactate concentrations at different time points. Data represents Mean±SEM (n=4)

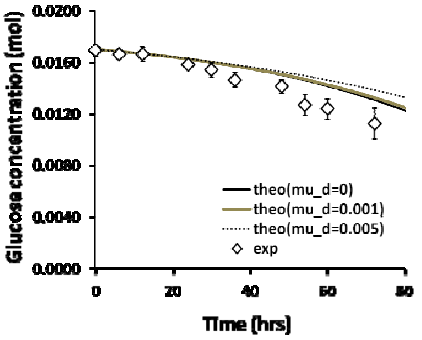

Fig. 6 Comparison of experimental glucose consumption with theoretical estimation at different cell death rates. Data represent Mean±SEM (n=4)

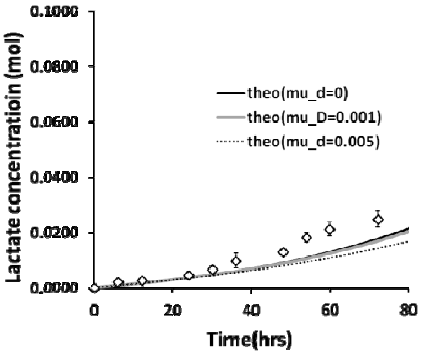

Fig. 7 Comparison of experimental lactate production with theoretical estimation at different cell death rate. Data represent Mean±SEM (n=4)

Similar to the trend observed with the viable cell count, increasing the value of μ_d from 0 to 0.005, decreased the total amount of glucose consumed as well as the total amount of lactate produced (as shown in Fig. 6 and Fig. 7, respectively).

A comparison of Figs 3, 6 and 7 also shows that incorporation of cell death value has more effect on the viable cell count rather than on glucose consumption or lactate production.

Although the theoretical curves fit the experimental data better at the initial stage of the experiments for the both the metabolite curves as well as the viable cell numbers, overall the simple mathematical model was able to predict the growth and metabolism trend of mouse embryonic fibroblast cells quite well. Hence the model can be used for similar analysis with other fibroblast cell lines which are used as feeder cells for pluripotent stem cells.

IV. Conclusion

The simple method of HPLC was used here for a metabolic analysis of SNL fibroblast cells. A theoretical fit was also made to support the consistency of the experimental data and help in extrapolating the data beyond the measured 72 hrs if and when needed.

A four fold increase in cell number was observed over a period of 72 hrs. Glucose concentration declined from 0.017 moles to 0.011 moles while lactate concentration increased to about 0.025 moles. The mathematical model was able to predict the data trend in all three cases. However variation between the experimental data and the theoretical fit increases at higher time points, highlighting the need for a more optimized model for the same.

The ability of the mathematical model to fit the current data, as well as its validity in a previous publication with mesenchymal stem cells means it is a useful model for metabolic profiling of similar cell lines. [15]

This metabolic analysis is expected to help in the proper use and better optimization of fibroblast conditioned media for *in vitro* expansion of pluripotent stem cells.

Appendix

Notations:

K_g= Monod constant of glucose (mol)

k_{md} = Degradation rate constant of metabolite (hr^{-1})

q_g=Specific consumption rate of glucose (mol/cell/hr)

q_{gl}= Specific consumption rate of glutamine (mol/cell/hr)

q_l= Specific production rate of lactate (mol/cell/hr)

q_m= Specific metabolite reaction rate (mol/cell/hr)

q_1 = Cell number at time t_1

q_2 = Cell number at time t_2

t_d= doubling time (hr)

X_g= Total moles of glucose in total volume (mol)

X_l= Total moles of lactate in total volume (mol)

X_m= Total moles of a metabolite in total volume (mol)

Where m = Metabolite referring to glucose or lactate

X_v = Total cell number in total volume (cells)

μ= Specific growth rate (hr^{-1})

μ_d= Death rate (hr^{-1})

μ_{max} = Maximum specific growth rate (hr^{-1})

Initial Values:

$X_v(initial)$= $7x10^4$

$X_g(initial)$= 0.017

$X_l(initial)$= $1.6x\ 10^{-4}$

μ_{max} = 0.01925

t_d= 36 hrs

q_g= $3.65x\ 10^{-10}$

q_l= $1.64x10^{-9}$

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