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Experimental analysis and modeling of in vitro mesenchymal stem cells proliferation

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Stem cell therapies based on the differentiation of adult or embryonic cells into specialized ones appear to be effective for treating several human diseases. This work addresses the mathematical simulation of the proliferation kinetics of stem cells. Sheep bone marrow mesenchymal cells seeded at different initial concentrations in Petri dishes are expanded up to confluence. The sigmoidal temporal profiles of total counts obtained through classic hemocytometry are quantitatively interpreted by a novel model based on a 1-D, single-staged population balance approach capable to take into account the contact inhibition at confluence. The models' parameters have been determined by comparison with experimental data on population expansion starting from a single seeding concentration. The reliability of the model is tested by predicting the cell proliferation carried out starting from different seeding concentrations.

1. Introduction

Transplantation procedures represent a suitable technique when restoration of native tissues and organs is impossible (Sarraf and Eastwood, 2005). In this framework, current research is focused on the use of stem cells, which, once proliferated *in vitro*, may be implanted, to regenerate *in vivo*, as faster as possible, the damaged tissue. Specifically, autologous stem cells may be obtained from tissues (skin, muscle, retina, neural, liver, intestine, mammary glands and others) of individual patients so that reimplantation of *in vitro* cultivated cells/tissues would avoid rejection problems. In particular, mesenchymal stem cells (MSCs) are multipotent stem cells, residing mainly in bone marrow (BM), which can be harvested from organs and cord blood, grown in culture as a homogeneous adherent population of fibroblast-like cells, and induced to differentiate into multiple cell types (cf. Pittenger *et al.*, 1999). However, there is only limited information on the optimization of culture conditions required for their production. To properly stimulate these cells to expand up to a sufficient amount for transplantation, the kinetics of their proliferation during *in vitro* cultivation is needed.

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The evaluation of the intrinsic kinetics of cell proliferation may be performed using static cultivation system (i.e. Petri dishes). The interpretation and analysis of the corresponding experiments, which provide a clear contribution to the understanding of the complex biological mechanisms involved in stem cell expansion/differentiation, may be achieved by means of suitable mathematical models.
In this work, our attention is focused on the kinetic study of ovine mesenchymal stem cells proliferation in static systems. In particular, the *ovine model* has been chosen as the cell system since it is more similar to the *human model* in terms of size and DNA than the classic *murine model*. The expansion kinetics is experimentally followed through classic hemocytometry for temporal evolution of total counts at different levels of seeding concentration. The proliferation kinetics is interpreted by means of a novel mass-based PBE model capable to simulate stem cell proliferation up to confluence in Petri dish due to contact inhibition, under the assumption of an excess of nutrients and oxygen supply. The model solutions were fitted to the temporal evolution of experimental total cell counts to determine the values of the model parameters and this was followed by prediction runs in order to test model reliability.

2. Materials and methods

After aspiration of 15 ml of bone marrow taken from the iliac crest of sheep, stem cells were first isolated by means of centrifiguation and by removing non-adherent blood cells after plating. Then, ovine bone-marrow derived stem cells were plated in 8-cm^2 petri dishes at a density of 1.25x10^3, 2.875x10^3 and 4.6x10^3 cells/cm^2, corresponding to 1.0x10^4, 2.3x10^4, and 3.68x10^4 total cells, respectively, and long term cultivated up to 14 days. Cells from three plates for each culture density were harvested day-by-day with the use of trypsin and EDTA and counted with a hemocytometer. The experiments were repeated at least three times. Finally, the performed phenotypic characterization by flowcytometric analysis ensured that differentiation during long term cultivation did not take place. On the basis of these results, it can be concluded that the sheep bone marrow stem cells considered in this work do not differentiate during long-term cultivation in Petri dish, and, thus, proliferation with monolayer tendency is the only biological phenomenon taking place during the performed experimental runs. Details of the experimental procedures adopted are reported elsewhere (Mancuso *et al.*, 2009).

3. Model Equations

The mathematical model proposed in the present work describes cell growth and proliferation (expansion by mitosis) during cultivation in batch systems by means of the following PBE:

$$\frac{\partial \psi(m,t)}{\partial t} + \frac{\partial \left[v\, \psi(m,t) \right]}{\partial m} = 2 \int_m^{\infty} \psi(m',t)\ \Gamma^M(m', C_{O_2})\, p(m,m')\ dm' - \psi(m,t)\ \Gamma^M(m, C_{O_2}) \tag{1}$$

along with the initial and boundary conditions

$$\psi(m,\ t) = \psi^0(m) \qquad \text{for} \quad t = 0 \quad \text{and} \quad \forall\, m \tag{2}$$

$$\psi(m,t)=0 \qquad \text{for} \quad t>0 \quad \text{and} \quad m=0 \tag{3}$$

Here $\psi(m,t)$ represents the number concentration density distribution of cell mass m at time t, which is assumed to be spatially uniform. In Equation (1) the two terms of the left-hand-side represent the accumulation and the cell growth term, respectively. On the other hand, the first term of the right-hand-side of Equation (1) represents the birth one (i.e. two daughters cells are obtained by division of a larger mother cell), while the second term represents the corresponding removal of mother cell due to mitosis.

The three generally unknown functionalities, namely cell mass growth rate (ν), cell division rate (Γ^M), and partitioning distribution of mother cell into daughters (p) needs to be specified. The latter one expresses the conditional cell mass density function for a daughter cell given the size of a mother cell at mitosis. In particular, following the approach described in detail in our previous work (cf. Pisu *et al.*, 2007), the unequal partitioning continuous distribution functionality is taken into account:

$$p(m,m')=\frac{1}{\beta(q,q)}\frac{1}{m'}\left(\frac{m}{m'}\right)^{q-1}\left(1-\frac{m}{m'}\right)^{q-1} \tag{4}$$

where the symmetrical beta function and the gamma function are adopted for $\beta(q,q)$ and $\Gamma(q)$, respectively (i.e. $\beta(q,q)=\frac{(\Gamma(q))^2}{\Gamma(2q)}$ and $\Gamma(q)=\int_0^{+\infty} s^{q-1}\,e^{-s}\,ds$).

The cell division rate Γ^M is determined from growth rate ν and the distribution of mitotic cells $f(m)$ by means of the following relationships:

$$\Gamma^M(m,C_{O_2})=\nu(m,C_{O_2})\cdot\gamma^M(m) \tag{5}$$

$$\gamma^M(m)=\frac{f(m)}{1-\int_0^m f(m')dm'} \tag{6}$$

$$f(m)=\frac{1}{\sqrt{2\pi\sigma^2}}\exp\frac{-(m-\mu)^2}{2\sigma^2} \tag{7}$$

where cell mass is used to predict cell division. In particular, in order to account for heterogeneity in cell cycle distribution, the function *f(m)* is represented by a normal distribution of dividing mass around a mean μ with variance σ^2 (i.e. cell division is more probable when cell reaches a critical mass μ).

Finally, the time rate of change of cell mass, ν , is expressed as reported below:

$$\nu(m,C_{O_2})=\left(\frac{3}{d_c}\right)^{2/3}(4\pi)^{1/3}m^{2/3}\frac{\mu'\,C_{O_2}}{C_m+C_{O_2}}\Phi-\mu_c m \tag{8}$$

where, the limiting supply of oxygen (nutrient) is taken into account by a classic Monod kinetics. However, the experimental procedure adopted in this work guarantees a constant concentration of nutrients supplied to the expanding culture, so that the

relevant oxygen material balance is not taken into account. Moreover, in order to simulate contact inhibition which progressively slows down culture expansion when reaching the confluence in the Petri dish as proliferation progresses, a limiting factor ($\Phi(t) \le 1$) appearing in the anabolic term is proposed. Assuming that cells tend to distribute themselves on a monolayer, the geometric limiting factor may defined through the following power-law:

$$\Phi(t) = \left[1 - \frac{\varphi(t)}{\varphi_a} \right]^{\alpha_p} \tag{9}$$

where $\varphi(t)$ represents the occupied area by cells and interstices (increasing with time as proliferation progresses), φ_a the available area (corresponding to the Petri dish area), and α_p the adjustable parameter. Accordingly, the occupied area by cells and their interstices is roughly calculated as the bidimesional projection of a monolayer culture of spherical cells as follows:

$$\varphi = V \left(\frac{4}{\pi} \right) \left(\frac{3\,\pi}{4\,d_c} \right)^{2/3} \int_0^{\infty} m^{2/3}\, \psi(m,t)\, dm \tag{10}$$

where the factor $4/\pi$ is the ratio between the surfaces of a square (i.e. cell and interstices) and the inscribed circle (i.e. cell) within it, while V is the cultivation volume, and d_c is cell mass density.

Equation (1) is a partial integro-differential equation in the variables *t* and *m* which has been solved by the method of lines (i.e. by discretization of *m* domain and temporal integration as an initial value problem of the resulting system of ordinary integro-differential equations through standard numerical libraries).

4. Results and discussion

The selected initial cell distribution is a log-normal distribution, $\psi^0(m) = \frac{(N^0/V)}{m\,\sigma^0\,\sqrt{2\,\pi}} \exp\left[-\frac{1}{2} \left(\frac{\ln(m) - \ln(\mu^0)}{\sigma^0} \right)^2 \right]$, where N^0 represents the total cell number initially seeded on the Petri dish. It is characterized by a mean value (μ^0) and a standard deviation (σ^0). The first one is assumed to be equal to 2 ng (cf. Table 1) which corresponds to the average, spherical cell having a diameter of about 15 μm, as experimentally evaluated in this work by microscopic analysis. Standard deviation, on the other hand, is chosen equal to 0.4 so that small daughter cells and large mother cells are both present in the seeded population. The initial cell mass distribution normalized with respect to the initial, total number of cells, is plotted in Figure 1a. Such distribution is used in all the simulations. Model parameters used for the simulations of the PBE modeling approach are reported in Table 1.

Table 1 Model parameters.

Parameter	Value	Unit
C_{O_2}	$0.203\text{x}10^{-6}$	mmol/mm^3
C_m	$0.006\text{x}10^{-6}$	mmol/mm^3
d_c	$1.14\text{x}10^{6}$	ng/mm^3
N^0	Run 1: $3.68\text{x}10^4$ Run 2: $2.3\text{x}10^4$ Run 3: $1.0\text{x}10^4$	cells
q	40	-
V	800	mm^3
α_P	10.8	-
φ_a	800	mm^2
μ	3.8	ng
μ^0	2	ng
μ_C	$1.0\text{x}10^{-3}$	1/h
μ'	97.4	ng/(mm^2 h)
σ	1.125	ng
σ^0	0.4	ng

In particular, two parameters have been determined using a nonlinear fitting procedure, i.e. the proportionality constant, μ', of cell mass growth rate (cf. Equation (8)), and the order α_P of the power law given in Equation (12). The others are taken from the literature or depends both on the operative conditions adopted in this work as well as on the specific cell lineage at hand (Mancuso *et al.*, 2009). The comparison between model and experimental data is illustrated in Figure 1b, where the evolution of the total number of cell, when starting from $3.68\text{x}10^4$ cells, is reported as a function of time along with the temporal profile of the geometric limiting factor $\Phi(t)$ defined in Equation (10).

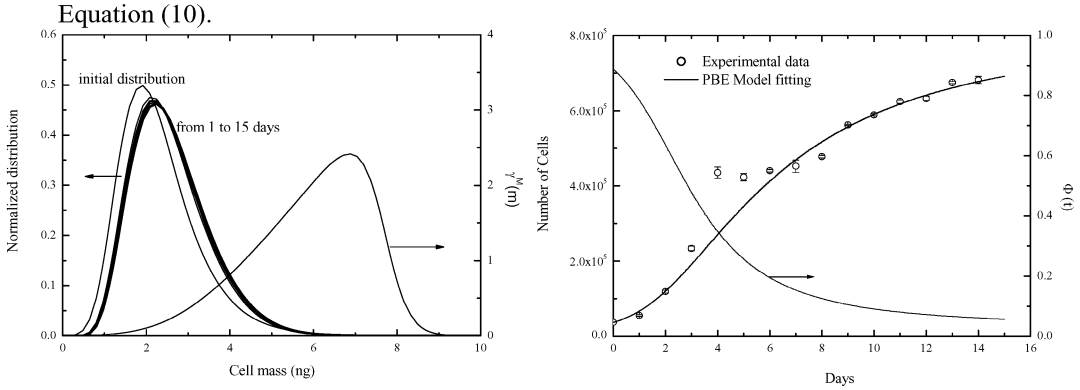

Figure 1 Normalized distributions at different cultivation times and $\gamma^M(m)$ distribution (a), comparison between model results and experimental data on total cell number, and geometric limiting factor $\Phi(t)$ (b) when starting with $3.68\text{x}10^4$ cells.

To test the predictive model capability, the experimental data obtained when cultivation is carried out by starting with $2.3x10^4$ and 10^4, respectively, as initial number of cells, are simulated by keeping constant the model parameters as previously determined and reported in Table 1. Figure 2 shows the comparisons of model results with these experimental runs.

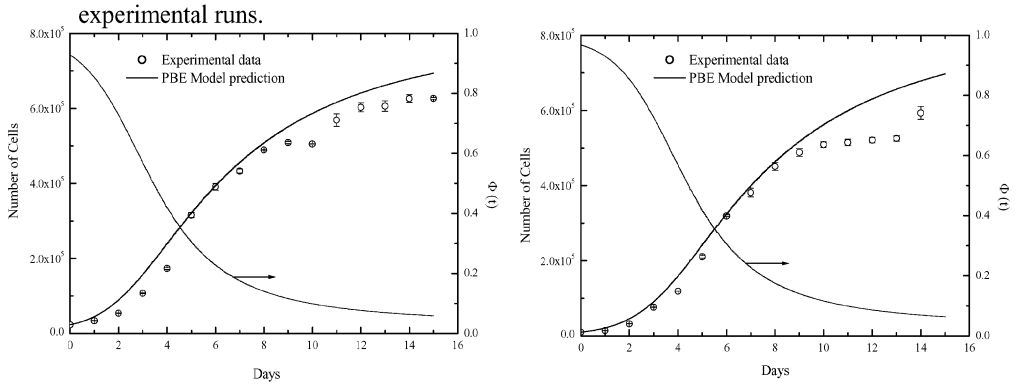

Figure 2 Comparison between model results and experimental on total cell number, and geometric limiting factor $\Phi(t)$ *when starting with* $2.3x10^4$ *cells (a) and* 10^4 *cells (b).*

As can be seen, the PBE acceptably predicts the temporal profile of the number of proliferating cells at varying seeding concentrations. In Figure 1a the normalized cell distribution at different cultivation times is reported for the case of an initial total cell number equal to $3.68x10^4$ cells along with the term $\gamma^M(m)$ which contains the effect on mitotic rate Γ^M (cf. Equations (5-6)) by the adopted functionality for the distribution of cells at mitosis (f(m)). As can be seen, the so-called state of "balanced growth" (i.e. time-invariant cell mass distribution) is practically reached in one day of cultivation and maintained throughout the proliferation runs. This is due to the significant overlapping between the initial cell and $\gamma^M(m)$ distributions, which reflects the presence of a mitotic fraction in the initial cell population seeded into the Petri dish.

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