Shear effects on suspended marine sponge cells

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Abstract

Fractions of viable cells, apoptotic and irreversibly damaged cells, dead whole cells and cell fragments were measured by flow cytometry during the production of freely suspended primary cells from explants of the marine sponge Axinella damicornis. The explants were disintegrated using the well-known Müller protocol [W.E.G. Müller, M. Wiens, R. Batel, R. Steffen, R. Borojevic, M.R. Custodio, Establishment of a primary cell culture from a sponge: primmorphs from Suberites domuncula, Mar. Ecol. Progr. Ser. 178 (1999) 205–219]. Supplementation of the standard Ca2+ and Mg2+-free artificial seawater of the Müller protocol, with the shear protectant Pluronic F68 (0.1%, w/v) greatly reduced the cell damage and enhanced the recovery of viable cells at each of the four stages of the protocol. Agitation of cells on an orbital shaker at 75 rpm essentially killed all the viable cells within 2.5 h, but no loss of viability occurred at a higher agitation speed of 100 rpm for up to 6 h when the cells were supplemented with Pluronic F68. This time-dependent loss in viability could be significantly reduced by processing at 3 °C instead of the normal 17 °C. A four-step mechanistic model was shown to describe the kinetics of cell death and fragmentation within ±10% of the measured values. The damage to cells was modeled as a web of first-order processes that did not depend on cell–cell interactions. The forces in the agitated fluid killed the viable cells by impact, which was not accompanied by cell rupture (i.e. the cell was left dead, but intact).

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I. Introduction

Marine sponges are increasingly being examined as possible sources of unusual bioactive compounds [1,2]. Sponge biomass for recovery of metabolites is generally obtained from explants grown in the sea and aquariums. Sponges cultivated by these methods are invariably contaminated with various microbial populations. In view of this, interest is emerging in producing metabolites using pure cultures of freely suspended sponge cells and cell aggregates (primmorphs). Protocols have been established for producing primary cell suspensions from sponge tissue [3], although no cell lines capable of indefinite growth have been obtained [2]. In many cases, the primary cells have been cultured and multiplied as primmorphs, or morphologically well-defined cell aggregates that form spontaneously from suspended cells [2,4,5].

In suspension culture, cells are exposed to hydrodynamic and mechanical forces that they do not normal encounter in the sponge matrix. Like most types of animal cells [6,7], freely suspended sponge cells are susceptible to damage by these forces, but no data exist on such effects. Here we show that the most commonly used method of generating primary cell suspensions from sponge tissue [3], causes damage to cells and provide an improved variant of the method to reduce the damage. The improved method combines the effect of temperature and the use of shear protective additive Pluronics F68 for enhancing cell survival. A mathematical model of cell damage is used to show that in media free of gas bubbles, damage to cells is a first-order process that does not depend on cell–cell interactions. This is consistent with other published observations [6,7]. The results are useful for possible future long-term suspension culture of sponge cells in bioreactors.
2. Materials and methods

Specimens of the marine sponge Axinella damicornis were collected by SCUBA divers in the Mediterranean coastal waters (Almería, Spain), immediately transported to the laboratory and maintained in an aquarium as described previously [8]. The sponge was kindly identified at Centro de Estudios Avanzados de Blanes (Girona, Spain).

<table>
<thead>
<tr>
<th>Greek letters</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>parameter defined in Eq. (13)</td>
</tr>
<tr>
<td>$\beta$</td>
<td>parameter defined in Eq. (14)</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>parameter defined in Eq. (15)</td>
</tr>
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</table>

The sponge was kindly identified at Centro de Estudios Avanzados de Blanes (Girona, Spain).

Steps 5 and 6 represent direct cellular disintegration from the initially viable state to some of the other possible states through a dynamic process, as schematized in Fig. 1a. The four steps in Fig. 1b are obviously irreversible. In keeping with prior evidence for various animal cell suspensions [6,7], we assume that each step is a first-order process, i.e. the damage is not due to collisions or other interactions among cells, but hydrodynamic shear forces that are independent of the cell concentration. Thus, for cells that do not grow, following steps 5 and 6 logically are close to zero, and the scheme of Fig. 1a is simplified to Fig. 1b. The four steps in Fig. 1b are obviously irreversible. In keeping with prior evidence for various animal cell suspensions [6,7], we assume that each step is a first-order process, i.e. the damage is not due to collisions or other interactions among cells, but hydrodynamic shear forces that are independent of the cell concentration. Thus, for cells that do not grow, following balances can be written for the fractions of viable, dead, apoptotic or irreversibly damaged cells and debris, respectively, as measured by flow cytometry.

In an agitated cell suspension the cells are transformed from the initially viable state to some of the other possible states through a dynamic process, as schematized in Fig. 1a. Steps 5 and 6 represent direct cellular disintegration from whole cells to debris when the bioreactor is intensely agitated (i.e. internal impeller and/or bubbling). When suspensions of animal cells are gently agitated like in this work (i.e. orbital shaker) constants $K_{1-4}$ logically are close to zero, and the scheme of Fig. 1a is simplified to Fig. 1b. The four steps in Fig. 1b are obviously irreversible. In keeping with prior evidence for various animal cell suspensions [6,7], we assume that each step is a first-order process, i.e. the damage is not due to collisions or other interactions among cells, but hydrodynamic shear forces that are independent of the cell concentration. Thus, for cells that do not grow, following balances can be written for the fractions of viable, dead, apoptotic or irreversibly damaged cells and debris, respectively, as measured by flow cytometry.

3. A model of shear damage

The total number of events ($N_T$) measured by the flow cytometer in a sample are made up of the following: (1) the number of viable cell events ($N_V$); (2) the dead cell events ($N_D$); (3) events associated with irreversibly damaged cells ($N_A$); (4) events associated with cell debris or fragments ($N_M$).

Thus, a total event balance can be written as follows:

$$N_T = N_V + N_A + N_D + N_M$$

Dividing Eq. (1) by $N_T$ and multiplying by 100, we obtain the event balance in percentage terms:

$$100 = \frac{N_V}{N_T} 100 + \frac{N_A}{N_T} 100 + \frac{N_D}{N_T} 100 + \frac{N_M}{N_T} 100$$

$$100 = V + M + D + A$$

where $V$, $M$, $A$ and $D$ are the percentages of viable cells, dead cells, apoptotic or irreversibly damaged cells and debris, respectively, as measured by flow cytometry.

$\frac{dV}{dt} = -(K_1 + K_3)V$

$\frac{dM}{dt} = K_1V - K_4M$

$\frac{dA}{dt} = K_2V - K_3A$

$\frac{dD}{dt} = K_3A + K_4M$

$\frac{dN}{dt} = -dV - dM - dA - dD$
Fig. 1. A model for the emergence of apoptotic cells, irreversibly damaged cells, dead cells and cell debris from viable cells. The first-order rate constants for the various steps are \( K_i \) for the steps A to M (Fig. 1b), i.e. whole cells may be directly disintegrated \((K_{i,0} \neq 0)\); (b) whole cells are not directly disintegrated \((K_{i,0} = 0)\).

Simultaneously solving Eqs. (4)–(6), leads to the following equations:

\[
\frac{dA}{dt} = -K_1A + K_1V
\]

\[
\frac{dD}{dt} = -K_2D + M + K_4A
\]

where \( K_{1,0} \) are the first-order rate constants for the steps shown in Fig. 1b.

Simultaneously solving Eqs. (4)–(6), leads to the following equations:

\[
V = V_0 \exp\left(-(K_1 + K_3)t\right)
\]

\[
M = \frac{\alpha + \beta}{K_2 - K_1 - K_3} e^{-(K_1 + K_3)t} - \frac{\gamma}{K_2 - K_3} e^{K_3t} + M_0 e^{-K_4t}
\]

\[
A = A_0 - \frac{K_1V_0}{K_1 + K_3} e^{-(K_1 + K_3)t} - 1
\]

\[
D = 100 - V - M - A
\]

Assuming, logically, that the apoptotic or irreversibly damaged cells die slowly, the time scale for going from \( A \) to \( M \) (Fig. 1b) will be much larger than the time scales for the other steps in Fig. 1b, i.e. \( K_3 \ll K_1, K_2 \) (\( K_3 \) is close to zero). Thus, Eqs. (8) and (9) can be simplified to the following equations:

\[
M = \frac{\alpha}{K_2 - K_1 - K_3} e^{-(K_1 + K_3)t} - \frac{\gamma}{K_3} e^{K_3t} + M_0 e^{-K_4t}
\]

\[
A = A_0 - \frac{K_1V_0}{K_1 + K_3} e^{-(K_1 + K_3)t} - 1
\]

where \( \alpha = K_1V_0 \)

\[
\beta = \frac{K_4K_3V_0}{K_4 - K_1 - K_3}
\]

\[
\gamma = K_4A_0
\]

In the above equations, \( V_0 \) is the initial percentage of viable cells, \( M_0 \) is the initial percentage of dead cells, \( A_0 \) is the initial percentage of apoptotic or irreversibly damaged cells and \( D_0 \) is the initial percentage of cell debris. The constants \( K_i \) are likely to depend on factors such as temperature, agitation speed and the viscosity of the broth.

4. Results and discussion

The time profiles of the variables \( A, D, M \) and \( V \) are shown in Fig. 2 at the agitation intensities of 0 rpm (i.e. controls) and 75 rpm. Note that the x-axes of the two plots (Fig. 2) have different scales. In both cases, the fraction of the apoptotic or irreversibly damaged cells (i.e. \( A \)) is always low, but the viable fraction of the population (i.e. \( V \)) is lost much more rapidly in the agitated culture. The standard deviation and the average values of the measured variables show a high level of reproducibility of the measurements (Fig. 2).

To facilitate comparison, the data on viable cell fraction (Fig. 2) at the two agitation intensities are replotted in Fig. 3. Clearly, in the absence of agitation viable cells can be detected at up to 96 h, but fewer than half the original live cells have survived by 2 h in the agitated environment (Fig. 3). This is clear evidence of an exceptionally high sensitivity of the marine sponge cells to hydrodynamic forces.

The model-generated profiles and the experimental data for the variables \( V, A, D \) and \( M \) are shown in Fig. 4 at two values of the agitation speed. The figure demonstrates an excellent ability of the model to reproduce the measured values. Eqs. (7)–(10) are used to generate the various modeled profiles shown in Fig. 4. The viable fraction of the cells declined extremely rapidly at 100 rpm. A more gradual decline occurred under more gentle agitation of 75 rpm (Fig. 4). Interestingly, the fraction of the apoptotic or irreversibly damaged cells was always low throughout the experimental period and irrespective of the agitation intensity (Fig. 4). This suggests that the progression to apoptosis or irreversibly damage from the viable state did not depend on hydrodynamic conditions and the cells that attained this state remained in it a long time.
Unlike the fraction of the apoptotic or irreversibly damaged cells, the cell debris fraction \((D)\) rose with time. This and the profiles of \(M\) (i.e. dead whole cells) in Fig. 4 suggest that the viable cells were lost in two consecutive ways (steps 1 and 2 in Fig. 1b): (1) by being killed while remaining physically intact, by the damaging forces and (2) by being subsequently disintegrated into debris by physical breakage. The shape of the \(M\) and \(D\) profiles suggest that the second process was slower than the one that initially had caused the kill (i.e. \(K_1 > K_2\)).

The values of \(K_{1-3}\) that produced the best fit of the modeled profiles to the measured values of \(V, A, D\) and \(M\) are shown in Table 1 for the various agitation speeds. A negative value of \(K_3\) in one case may suggest that the viable-to-apoptotic or irreversible damage transition may not be completely irreversible, or simply that both reversible and irreversible damage to a cell are possible scenarios. The negative value cannot be ascribed to experimental error, as the values of \(A\) used in the fit were all within experimental error. The \(K_{1-3}\) values

Table 1

<table>
<thead>
<tr>
<th>(N) (rpm)</th>
<th>(K_1) (h(^{-1}))</th>
<th>(K_2) (h(^{-1}))</th>
<th>(K_3) (h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0225</td>
<td>0.0055</td>
<td>-0.0006</td>
</tr>
<tr>
<td>75</td>
<td>0.5096</td>
<td>0.0849</td>
<td>-0.0118</td>
</tr>
<tr>
<td>100</td>
<td>1.0810</td>
<td>0.1318</td>
<td>0.1095</td>
</tr>
</tbody>
</table>
(Table 1) confirm a nonlinear dependence of these rate constants on the hydrodynamic regimen (i.e. N-value).

For all experiments, the model predicted fraction of the viable cells consistently agreed with the measurements within ±10% for the full range of agitation intensities (including the nonagitated controls), as shown by the parity plots in Fig. 5.

This substantiates the general cell damage and debris generation mechanisms proposed in Fig. 1b and the following significant assumptions of model: (1) A first-order transformation of the viable cells to apoptotic or irreversibly damaged cells and dead cells; (2) a long-lived apoptotic or irreversible damage state that was essentially independent of the hydrodynamic conditions; (3) a first-order disintegration of the dead whole cells to fragments; (4) an absence of cell–cell interactions as a factor contributing to cell damage.

4.1. Effects of Pluronic® F68

The effects of supplementing the cells with Pluronic® F68 in suspensions agitated at 100 rpm, are shown in Fig. 6. In PF68-free suspension, the viable fraction (V) of the populations declined dramatically within 1 h of agitation and the fraction of the debris D rose with time. In contrast, the viable fraction and debris levels remained stable for up to 6 h in PF68-supplemented suspensions. Clearly, PF68 had a protective effect on cells.

Pluronic® F68 is a nonionic surfactant polyol with a molar mass of 8.3 kDa. The surfactant is not metabolized by animal cells and has no effect on the viscosity of suspensions in the...
Fig. 7. Percent of viable cells, dead cells, irreversibly damaged cells and debris in samples at various times. Solid bars are for controls (no. PF68). Grey bars are for samples supplemented with PF68.

concentration used. PF68 has been shown to have a protective effect on a great variety of animal cells in both aerated and un aerated cultures. Because it does not affect viscosity, a possible dampening of turbulence in PF68-supplemented media cannot be an explanation for the observed cell protective effect. PF68 is known to biochemically associate with the cell membrane to reduce the plasma-membrane fluidity and this has been suggested as a possible mechanism of protection [6,10–14]. A physical strengthening of the cells will almost certainly reduce the values of the first-order rate constants $K_{1-3}$ in Fig. 1, to reduce the cell damage.

The Müller protocol for producing primary cell suspensions from sponge tissue, typically requires agitation of the tissue in CMFSW with a change of the suspending medium at 40, 80, 160 min and 24 h intervals [3]. The protocol as used currently, does not supplement the medium with PF68. Results presented in Fig. 7 show that in comparison with the current protocol, supplementation with PF68 greatly improves the yield of the viable cells and reduces the fraction of the dead whole cells at each of the transfer stages (0.67, 1.33, 2.7 and 24 h) of the protocol. Thus, PF68 reduces damage to cells as they are produced by dissociation from the parent sponge tissue.

4.2. Effects of incubation temperature

The effect of incubation temperature (3 and 17°C) on survival of suspended cells in nonagitated medium was examined in some experiments. The data on viable fraction $V$ and the ratio of the viable to dead whole cells ($V/M$), are shown as a function of incubation time and temperature in Fig. 8. Although in the first 2.5 h, the effect of temperature on decline in the viable population is not quite obvious, the measurements at 96 h clearly reveal that the viable fraction of the population declined much slower at 3°C than at 17°C (Fig. 8). Furthermore, at the lower temperature the viable cells constituted a much bigger proportion of the intact cells than at the higher temperature (Fig. 8). The results confirm that the rate constants $K_{1-4}$ are temperature dependent in addition to being influenced by the hydrodynamic environment.
5. Concluding remarks

The following specific conclusions can be drawn:

1. The well-known Müller protocol [3] for producing viable sponge cells from explants, damages cells. The damage can be prevented by supplementing the artificial seawater medium with Pluronic® F68 at a concentration of 0.1 (w/v). Consequently, supplementation with PF68 can enhance the supply of cells for producing primmorphs and whole sponges from single cells.

2. Potentially, the damage to cells can be reduced by reducing the operating temperature.

3. Only a few apoptotic or irreversibly damaged cells are formed from viable cells and any apoptotic or irreversibly damaged cells formed, slowly progress to the dead cell stage.

4. A six-step mechanistic model was reduced to one of four-step for cell damage in orbital shakers, which explains exceptionally well the observed kinetics of damage. The rate of damage increases strongly with increasing intensity of agitation. The damage does not depend on cell–cell interactions and the individual steps of the model obey first-order kinetics.

Acknowledgements

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References