Analysis of cell accumulation mechanism in a rotational culture system

Koji Fukagata,† Katsuko S. Furukawa,‡ and Takashi Ushida‡


Abstract

The accumulation mechanism of cells in a rotational culture device is investigated from the viewpoint of fluid mechanics. For simplicity, the deformation of the water surface is neglected and the cells are treated as spherical solid particles. From the numerical simulation of flow field with typical parameters used in the previous experiments, it is confirmed that the relative velocity of fluid induced by the rotational shaking is much smaller than the speed of rotation. From the analysis of particle equation of motion, it is found that the accumulation of cells toward the central region is found to be due to interaction between the acceleration by rotational shaking and the drag force acting on the cells. The integral time scale for cell accumulation was estimated to be about 10 minutes for typical cases. The accumulation speed increases quadratically with the diameter of cell and the angular velocity of rotational shaking, which qualitatively support the previous experimental observation.

1 Introduction

In tissue engineering, it is often required to have aggregation of large number of primary cells in order to improve their functions. Although the pellet culture system is widely used, it is hardly applicable to the clinical application due to the insufficient number of cells that can be aggregated.

As an alternative method, the rotational culture attracts increasing attention for improving the aggregation rate. A typical rotational culture system is composed of a dish containing the primary cells immersed in fluid and a rotary shaker that rotationally shakes the dish. Recent experiments clearly demonstrate that different kinds of cells can be efficiently aggregated by the rotational culture system. The present experiment (of which process is briefly overviewed in the next section) also confirms that the initially dispersed cells develop into a large three-dimensional aggregate as shown in Fig. 1. The cause for this enhanced aggregation is observed to be due to accumulation of independent cells in the central region of the container followed by their coagulation. The size of aggregate is observed to be larger with slower, smaller with faster

†Corresponding author. Department of Mechanical Engineering, Keio University, Hiyoshi 3-14-1, Kohoku-ku, Yokohama 223-8522, Japan. E-mail: fukagata@mech.keio.ac.jp
‡Department of Bioengineering, Department of Mechanical Engineering, School of Engineering, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-8656, Japan.
‡Division of Biomedical Materials and Systems, Center for Disease Biology and Integrative Medicine, School of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan.
rotational shaking. This observation suggests that the time scale of accumulation is decreased with increase of the speed of rotational shaking.

Although the accumulation of individual cells is experimentally observed, its physical mechanism seems to be unclear. Therefore, in the present study, we attempt to clarify the fundamental mechanism of the local accumulation of cells from the viewpoint of fluid mechanics. For that purpose, numerical simulation and theoretical analysis are performed under a simplified condition. By clarifying the mechanism, one can design better rotational culture systems and their operating conditions.

2 Material and Methods

In this section, we briefly overview the procedure of experiment in order to make clear the target of present analysis. Readers mainly interested in the fluid mechanics may skip this section.

2.1 Cell Culture

Full-thickness articular cartilage was harvested aseptically from the femoropatellar grooves of canine calves within 24 hours of slaughter. The joints were exposed under aseptic conditions and the cartilage was sliced and minced with a scalpel blade. The fragments were rinsed 3 times with cold phosphate buffered saline (PBS) and digested with 0.15% collagenase Cl (Worthington Biochemical Corp, USA) in Ham’s F12 medium (Life Technologies, Japan) overnight on a rocking shaker at 37\degree C. Following filtration with a nylon mesh (70 \( \mu \)m cell strainers, Becton Dickinson Labware, USA), the cell suspensions were centrifuged for 10 min at 1500 \( g \) (\( \approx \) 14700 \( m/s^2 \)). The washed pellets were resuspended in medium consisting of Ham’s F12 (Life Technology, Japan), 10% fetal bovine serum (Gibco BRL, USA), 5 ng/ml fibroblast growth factor, 100 U/ml penicillin, 100 \( \mu \)g/ml streptomycin (Gibco BRL, USA), and 0.5 \( \mu \)g/ml fungizone. Chondrocytes were inoculated on dishes at a concentration of \( 6 \times 10^4 \) cells/cm\(^2\) in a humidified 37\degree C/5\% CO\(_2\) incubator. After approximately 1 week, first passage (P1) cells were detached using 0.25% trypsin/1.0 mM EDTA and replated at 1000 cells/cm\(^2\). Monolayer cultures were serially passaged three or four times as follows. A 500 cm\(^2\) square dish was seeded with chondrocytes at a density of 500 cells/cm\(^2\) in 70 cm\(^3\) of culture medium with 5 ng/ml fibroblast growth factor-2 (FGF-2, Peprotech, UK). Because it has been reported that bovine calf articular chondrocytes expand two-dimensionally in the presence of FGF-2 fully redifferentiated and deposited collagen type II in 3D culture conditions, we also added FGF to the growth

![](image)

Figure 1: Aggregates obtained by 24 hours of rotational culture at different angular velocities: (a) 70 rpm; (b) 80 rpm.
medium, but not to the differentiation medium. When the monolayer reached about 70% confluence, cells were removed after incubation with 0.25% trypsin and 0.1% EDTA for a few minutes. Then, the cells were centrifuged at 200 g (≈ 1960 m/s²) for 5 minutes, washed with calcium- and magnesium-free PBS, resuspended in fresh medium, and reseeded onto new square dishes.

2.2 Aggregate Formation

Single prepared cells of canine articular chondrocytes isolated from the femoropatellar grooves after 2nd passage were resuspended at 1.2 × 10⁶ cells/ml with the chondrogenic differentiation medium for rotational culture. The cells were inoculated into 6-well dishes with 5 ml of the chondrogenic medium, high-glucose (4.5g/l) Dulbecco modified Eagle medium (DMEM, Gibco BRL, USA), supplemented with 100 nM dexamethasone (Sigma, USA), 50 µg/ml ascorbic acid 2-phosphate (WAKO Pure Chemicals, Japan), 100 µg/ml sodium pyruvate, 40 µg/ml proline, and ITS-plus (Collaborative Biomedical Products, USA, final concentrations, 6.25 µg/ml bovine insulin, 6.25 µg/ml transferrin, 6.25 µg/ml selenous acid, 5.33 µg/ml linoleic acid, and 1.25 mg/ml bovine serum albumin), penicillin, streptomycin, fungizone and cultured under a dynamic condition of a rotational culture at the speed of 70 and 80 rpm. The dishes were placed in a humidified CO₂ incubator.

3 Numerical Simulation of Fluid Velocity Field

3.1 Description of System

We consider a cylindrical container of R in inner radius and water filled in it, of which height is H, as shown in Fig. 2. For simplicity, we assume an isothermal and incompressible fluid. Moreover, we neglect the deformation of water surface. The container orbits around a fixed point at the angular velocity of Ω. The distance of between two centers is denoted as A.

3.2 Governing Equations

In order to simplify the analysis, we choose observation frames translating with the motion of the container. In addition to the translation, one can arbitrarily choose the angular velocity, ω, of the observation frame. Two important cases are illustrated in

![Figure 2: Schematic of the cylindrical container subjected to the rotational shaking.](image-url)
Figure 3: Two important angular velocities of the coordinate system for observation. (a) $\omega = 0$; (b) $\omega = \Omega$. Times $t = t_1$ and $t = t_2$ denote two different times.

Fig. 3. With $\omega = 0$, the observation frame is simply translating with the motion of container and is not subjected to rotation. The relation between any physical point of the container, e.g., point P in Fig. 3, and the observation frame is unchanged. The velocity on the side wall is observed to be zero. The direction of centrifugal force due to the rotational shaking, however, is observed to change in time; thus it complicates the analysis. With $\omega = \Omega$, on the other hand, the observation frame is rotating. The velocity on the side wall is observed to be $u_q = -\Omega R$. The centrifugal force due to the rotational shaking is observed in a fixed direction, i.e., $\theta = 0$. Since the boundary condition is steady, the problem may be treated as a steady flow problem, at least when the Reynolds number is sufficiently low.

On the observation frame that translates with the motion of container and rotates at the angular velocity of $\omega$, the equations of motion for the fluid in the container, of which density is $\rho$ and kinematic viscosity is $\nu$, can be given by the continuity equation,

$$\nabla \cdot \mathbf{u} = 0,$$  \hspace{1cm} (1)

and the momentum equation,

$$\frac{\partial \mathbf{u}}{\partial t} = -\nabla \cdot (\mathbf{u} \mathbf{u}) - \frac{\mathbf{V} p}{\rho} + \nu \nabla^2 \mathbf{u} + \ddot{a}.$$  \hspace{1cm} (2)

where $\mathbf{u}$ and $p$ denote the velocity and pressure, respectively. Considering the cylindrical coordinates on the observation frame ($\mathbf{e}_r, \mathbf{e}_\theta, \mathbf{e}_z$), the additional superficial force to the Navier-Stokes equation, $\ddot{a}$, can be expressed as

$$\ddot{a} = \left( \begin{array}{c} a_r \\ a_\theta \\ a_z \end{array} \right) = \left( \begin{array}{c} -\Lambda \Omega^2 \cos \theta + (\omega - \Omega) t \\ -\Lambda \Omega^2 \sin \theta + (\omega - \Omega) t \\ 0 \end{array} \right) + \left( \begin{array}{c} 2 \omega \Omega \theta \\ -2 \omega \theta \\ 0 \end{array} \right) - \nabla \phi.$$  \hspace{1cm} (3)

The first term in the right-hand-side is the the centrifugal force due to the rotational shaking, the second term represents the Coriolis force and the last term is the centrifugal force due to the rotation of the frame. Here, the scalar potential is defined as $\phi = \Omega^2 r^2/2$. 
The velocity boundary condition is no-slip on the side and bottom walls, i.e.,
\[
\begin{align*}
    u_\theta(R, \theta, z, t) &= -\omega R, \\
    u_r(R, \theta, z, t) &= u_z(R, \theta, z, t) = 0
\end{align*}
\]
and
\[
\begin{align*}
    u_\theta(r, \theta, 0, t) &= -\omega r, \\
    u_r(r, \theta, 0, t) &= u_z(r, \theta, 0, t) = 0.
\end{align*}
\]
Free-slip boundary condition is adopted on the water surface, i.e.,
\[
\begin{align*}
    \frac{\partial u_r}{\partial z}(r, \theta, H, t) &= \frac{\partial u_\theta}{\partial z}(r, \theta, H, t) = \frac{\partial u_z}{\partial z}(r, \theta, H, t) = 0.
\end{align*}
\]
A Reynolds number can be defined as
\[
    Re_R = \frac{R^2 \Omega}{\sqrt{\nu}}.
\]
There are three independent parameters dominating the flow, i.e., \(A/R, H/R\) and \(Re_R\). In addition, the flow field looks differently depending on the choice of the observation frame: \(\omega = 0\) and \(\omega = \Omega\) correspond to Figs. 3(a) and (b) respectively.

### 3.3 Numerical Procedure

The governing equations is spatially discretized by using the energy conservative second-order accurate finite difference scheme. The computational mesh is uniform in each direction. The number of mesh points is \(96 \times 32 \times 16\) in the radial \((r)\), azimuthal \((\theta)\) and longitudinal \((z)\) directions, respectively. In the computation, all the quantities are made dimensionless by using the container radius, \(R\), and the angular velocity of shaking, \(\Omega\).

The temporal integration is done by the third-order accurate low-storage Runge-Kutta/Crank-Nicolson scheme. The computational time step is \(2 \times 10^{-3} \pi \Omega^{-1}\). The velocity-pressure coupling is treated by using a delta-form fractional step method. The Poisson equation for the correction of velocity and pressure is solved by using two-dimensional (i.e., \(\theta\) and \(z\)) trigonometric expansion with the mirroring technique applied in the non-periodic \((z)\) direction.

In the numerical simulation, the observation frame rotating at \(\omega = \Omega\) is chosen because the superficial acceleration is unidirectional on that frame (as mentioned above) and thus the flow field is expected to reach a stationary state after a long time. On the other hand, most results are presented on the non-rotating frame \(\omega = 0\), which is intuitively easier to understand than those expressed on the rotating frame.

### 3.4 Simulation Results

Based on the typical parameters used in the experiments (Table 3.4), the non-dimensional parameters for the present computation are chosen as shown in Table 3.4.

The simulation is conducted for a period of \(T = 200\Omega^{-1}\); the velocity field is found to reach a stationary state as we expected. Figure 4 shows the \(r-\theta\) cross-sectional velocity vectors on the water surface (i.e., \(z = H\)) at two different speeds of rotational shaking, \(Re_R = 2600\) and \(5200\), which correspond to 70 and 140 rpm in the experimental conditions, respectively. The observation frame is non-rotational (\(\omega = 0\)) and the
The velocity field shown is the snapshot at the phase of $\theta = 0$. At this phase, the centrifugal force of the rotational shaking is working in the direction of positive $x$ axis. A weakly swirling flow is induced in nearly the same direction as the rotational shaking, with slight offset of the swirl center and some reverse flow near the right wall. The swirling is stronger for higher speed of rotational shaking. The magnitude of the azimuthal velocity is found to be $10^{-3}R\Omega$ at $Re_R = 5200$, which is negligibly small as compared to the speed of rotational shaking, $A\Omega$.

Figure 5 shows the radial velocity averaged in cross-section, $<u_r>$, defined as

$$<u_r>(z) = \frac{1}{\pi R^2} \int_0^R \int_0^{2\pi} u_r(r,\theta,z)r\,d\theta\,dr.$$  

At all the speeds of rotational shaking examined here, the cross-sectional radial velocity takes positive value near the water surface ($z/R = 0.3$) and negative value near the bottom wall ($z/R = 0$). This indicates that near the water surface outward mean flow is induced due to the external force, while near the bottom wall inward mean flow is induced so as to satisfy the global continuity. The magnitude of radial velocity is about 1% of the azimuthal velocity.

From the results presented above, it is confirmed that the induced flow is mainly directed to the azimuthal direction. The fluid velocity, however, is much smaller than the velocity of rotational shaking within the range of parameters examined here. The

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R$</td>
<td>17.5 mm (i.e., 35 mm in diameter)</td>
</tr>
<tr>
<td>$A$</td>
<td>12.7 mm (1 inch diameter orbit)</td>
</tr>
<tr>
<td>$H$</td>
<td>5 mm</td>
</tr>
<tr>
<td>$\Omega$</td>
<td>$8.4 , \text{s}^{-1}$ (70 rpm)</td>
</tr>
</tbody>
</table>
Dimensionless parameters used in the present simulation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( A/R )</td>
<td>0.7</td>
</tr>
<tr>
<td>( H/R )</td>
<td>0.3</td>
</tr>
<tr>
<td>( Re_R )</td>
<td>650 (18 rpm) – 5200 (140 rpm)</td>
</tr>
</tbody>
</table>

Figure 5: Radial velocity averaged in cross-section, \(< u_r >\) as the function of \( z \).

flow in \( r-z \) cross-section is even weaker.

4 Analysis of Cell Motion

In order to simplify the problem, we make the following idealization: (1) a cell is treated as a solid sphere; (2) the cell is passive and it does not influence the fluid motion (i.e., one-way coupling); and (3) cell-cell interaction is neglected. The motion of such a solid sphere can be described by the particle equation of motion by Maxey & Riley.\(^{14}\)

In the case of a small particle in water, however, one may usually neglect all the terms except for the Stokes drag. Thus, by denoting the particle velocity as \( \vec{v} = (v_r, v_0, v_z) \), the simplified particle equation of motion in this case reads

\[
\frac{d}{dt} \begin{pmatrix} v_r \\ v_0 \\ v_z \end{pmatrix} = \beta \begin{pmatrix} u_r - v_r \\ u_0 - v_0 \\ u_z - v_z \end{pmatrix} + 2\omega \begin{pmatrix} v_0 \\ -v_r \\ 0 \end{pmatrix} + A\Omega^2 \begin{pmatrix} \cos[\theta + (\omega - \Omega)r] \\ -\sin[\theta + (\omega - \Omega)r] \\ 0 \end{pmatrix} + \frac{-\omega^2 r}{0 0} \end{pmatrix}
\]

(9)

The coefficient \( \beta \) is the inverse of the Stokes particle relaxation time, \( \tau_p \), i.e.,

\[
\tau_p = \frac{d^2 \rho_p}{18 \nu \rho_f}.
\]

(10)
where \( d \) is the diameter of particle (i.e., cell), and \( \rho_f \) and \( \rho_p \) are the density of fluid and particle, respectively. The first term of the right hand side of the equations above represents the Stokes drag, the second term is the Coriolis force term and the third and fourth terms express the centrifugal forces due, respectively, to the rotational shaking and the rotation of observation frame.

When the relaxation time of particle is much smaller than the time scale of the fluid motion, i.e., \( \beta \gg \Omega \), the acceleration can be neglected, i.e., \( d/dt = 0 \). Thus, the particle equation of motion reads

\[
\begin{pmatrix}
\beta & -2\omega & 0 \\
2\omega & \beta & 0 \\
0 & 0 & \beta
\end{pmatrix}
\begin{pmatrix}
v_r \\
v_\theta \\
v_z
\end{pmatrix}
= \begin{pmatrix}
u_r \\
u_\theta \\
u_z
\end{pmatrix} + \beta \frac{\Omega^2}{2} \begin{pmatrix}
\cos[\theta + (\omega - \Omega)t] \\
-\sin[\theta + (\omega - \Omega)t] \\
0
\end{pmatrix}
+ \begin{pmatrix}
-\omega^2 r \\
0 \\
0
\end{pmatrix}.
\]

(11)

It is obvious that \( v_z = u_z \). The matrix equation for \( v_r \) and \( v_\theta \) components can also easily
Figure 7: Contours of the radial velocity ($v_r$) of $d = 15 \mu m$ particles at different height positions in $Re_R = 5200$ (140 rpm) case. (a) near the bottom ($z/R \simeq 0.01$); (b) at the height of maximum averaged inward fluid velocity ($z/R \simeq 0.08$). Increment of the contours is $1 \times 10^{-4}R\Omega$. Negative contours are dashed and the zero contour is in bold.

be solved to yield

$$
\begin{aligned}
\nu_r &= \frac{1}{\beta^2 + (2\omega)^2} \left( \beta^2 u_r + 2\beta \omega u_\theta - \beta \omega^2 r 
+ A \beta \Omega^2 \cos[\theta + (\omega - \Omega)t] 
- 2\alpha \omega \Omega^2 \sin[\theta + (\omega - \Omega)t] \right),
\nu_\theta &= \frac{1}{\beta^2 + (2\omega)^2} \left( \beta^2 u_\theta - 2\beta \omega u_r + 2\omega^3 r 
- A \beta \Omega^2 \sin[\theta + (\omega - \Omega)t] 
- 2\alpha \omega \Omega^2 \cos[\theta + (\omega - \Omega)t] \right).
\end{aligned}
$$

On the non-rotational frame ($\omega = 0$), Eq. (12) reduces to read

$$
\begin{aligned}
\nu_r &= u_r + A \tau_p \Omega^2 \cos[\theta - \Omega t], \\
\nu_\theta &= u_\theta - A \tau_p \Omega^2 \sin[\theta - \Omega t].
\end{aligned}
$$

On the rotational frame at $\omega = \Omega$, the time dependent term in Eq. (12) vanishes to read

$$
\begin{aligned}
\nu_r &\simeq u_r + \frac{\Omega}{\beta} \left( 2u_\theta - \Omega r + A\Omega \cos \theta \right), \\
\nu_\theta &\simeq u_\theta - \frac{\Omega}{\beta} \left( 2u_r - 2\Omega r + A\Omega \sin \theta \right).
\end{aligned}
$$
Here, we used the assumption that the particle relaxation time is much smaller than the time scale of rotational shaking, i.e., $\beta \gg \Omega$.

Figure 6 shows the contour of the computed radial velocity, $v_r$, of $d = 15 \mu m$ cells near the water surface at different speeds of rotational shaking. It can be observed that the radial velocity is negative (i.e., inward) in larger part. A region of positive (i.e., outward) radial velocity is found off the center. At higher speed of rotational shaking, and the larger negative velocity is found and the area of positive velocity is observed to gradually shrink. The distribution of radial cell velocity slightly depends on the height position in the vertical direction, especially when the rotation speed is high. Figure 7 shows the dependency of the radial cell velocity distribution on the height position in the case of $Re_R = 5200$ (i.e., 140 rpm). Although the shape of the outward velocity region slightly varies with the height, the velocity magnitude is found to be nearly unchanged.

The cross-section averaged inward velocities of fluid, $-\langle u_r \rangle$, and particles, $-\langle v_r \rangle$, near the water surface are plotted in Fig. 8. We used the values in Table 3.4 and a typical value of particle relaxation time, $\tau_p = 25 \times 10^{-5}$ s, which corresponds to $d = 15 \mu m$ non-buoyant cell in water. This shows that the particle velocity is much larger than the fluid velocity and it significantly increases with the increase of the speed of rotational shaking.

Taking into account that the azimuthal velocity on the rotational-frame ($\omega = \Omega$) is approximated as $u_\theta \simeq -\Omega r$ and that the radial fluid velocity is much smaller than the particle velocity, as shown in Fig. 8, the cross-section averaged inward particle velocity is approximated as

$$-\langle v_r \rangle \simeq \frac{1}{\pi R^2} \int_0^R \int_0^{2\pi} \tau_p \Omega^2 (A \cos \theta - 3r) r d\theta dr = 2R \tau_p \Omega^2.$$ \hspace{1cm} (15)

This approximated particle velocity is also plotted in Fig. 8, showing good agreement with that obtained from the numerical simulation. This result, that the cross-section averaged radial velocity of particles has little dependency on the fluid velocity, also
implying that it has little dependency on the height position. The simulation result shown in Fig. 9 supports this argument, which indicates that the variation is about 15% even at the highest speed considered here. Equation (15) suggests that the average inward velocity increases linearly with the radius of the container, $R$, and the particle relaxation time, $\tau_p$, and quadratic with angular velocity of rotational shaking, $\Omega$.

The integral time scale, $T_a$, for cell accumulation can be defined by using the radius of the container and the cross-section averaged inward particle velocity, i.e.,

$$T_a = \frac{R}{-\langle v_r \rangle} \approx \frac{1}{2\tau_p \Omega^2}.$$  

The time scale computed by using the simulation result is plotted in Fig. 10. The accumulation time is shorter for larger cells, longer for smaller cells, as suggested by Eq. (16). The figure shows that relatively fast (ca. 10 min) accumulation can be achieved at the rotational speed larger than 70 rpm. This is also in fair quantitative agreement with the observation in the previous experiment, in which the cells started aggregating in a few hours (Furukawa et al., 2003), considering the sequence that the accumulation of cells should occur before their aggregation. Observation in the previous experiment has been made only for relatively long time scale (~ hour), while the present analysis suggests that accumulation time scale is even shorter (~ 10 min). Exact comparison with an experimental measurement in shorter time scale is left for the future work.

5 Conclusions

The mechanism of the cell accumulation in an idealized cylindrical container subjected to a rotational shaking is investigated by means of numerical simulation and theore-
Figure 10: Integral time scale of cell accumulation as function of the speed of rotational shaking.

The present conclusions, however, should be applicable only under the simplified conditions assumed here. For instance, it is expected that the aggregated cells, which have larger diameter than assumed, have considerable influence on the flow field and interaction among cells may modifies their velocities. Moreover, the increase in diameter (of the aggregated cells) further accelerates their accumulation. Thus, the actual time scale of accumulation is expected to be shorter in than the present estimation. More accurate estimation may be viable only by numerical simulation including realistic cell models and their aggregation, which is left for the future work.

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