A linear shear model of cell viability loss during hepatocyte transplantation

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Abstract
Improving the process of cell injection during hepatocyte transplantation requires an understanding of the causal relationships that shear, direct contact cells with a solid surface, and cell deformation have on cell viability loss. A linear shear model was used to model this loss of cell viability during their movement on a solid surface as part of the injection step of hepatocyte transplantation. Rat hepatocytes were studied under linear shear using two parallel plates, with a "tight" condition that had a 25 µm gap, and a "loose" gap condition with a > 25 µm gap, to determine the effects of cell deformation, and simulate cell viability loss during injection. Cell morphology and deformation were also observed using time-lapse images. Direct contact with a solid surface is deleterious for cells, and live cells became deformed under shear stress until they lost viability. The cell size could decrease or increase during deformation, and a loss of viability could occur due to a loss of membrane integrity or cell rupture. The space limitations in the tight gap could prevent cell expansion, which delayed the process of cell viability loss. In summary, preventing the direct contact of hepatocytes with a solid surface is recommended to improve the cell injection process during transplantation.

Keywords : Cell viability loss, Simple shear, Cell deformation, Cell shrinkage, Cell expansion, Accidental cell death, Mechanically induced accidental cell death

1. Introduction
Cell viability loss within the delivery line of a needle or a catheter, along with sedimentation in the syringe, are physical problems that can occur during hepatocyte transplantation. Sedimentation during hepatocyte transplantation can usually be avoided by rotating or gently shaking the syringe (Meyburg et al., 2009a). However, the loss of viability in human hepatocytes during the cell injection process has been investigated using catheters with various sizes and flow rates, and it has been suggested that using a small catheter, 4.2 F, with a flow rate of 2 mL/min should be considered safe for clinical practice (Meyburg et al., 2009b). However, the shear stress experienced using the suggested conditions for 4.2 F was higher than with other catheters sizes at the same flow rate (Sufiandi et al., 2015). Based on this information, a macroscopic investigation was conducted to discover what happened to cells during the cell injection process using a microchannel model.

The effect of the injection process on cells was investigated by measuring cell viability using impermeable membrane markers to assess cell membrane integrity, along with cell apoptosis and caspase activity. Then, using a needle or catheter, the observations from pre- and post-injection cells were compared (Amer et al., 2015; Amer et al. 2016; Agashi, et al., 2009; Walker, et al., 2010; Meyburg et al., 2009b; Sufiandi et al. 2015). The loss of cell membrane integrity is observable as a stain by a membrane-impermeable marker. However, the exact process that leads to the loss of cell membrane integrity during the injection process has not been identified. While research exists on the characterization of the general mechanical properties of cells (Bao and Surresh, 2003; Huang et al., 2004; Lim et al., 2006; Lee and Lim, 2007; Addae-Mensah and Wikswo, 2008; Haase and Pelling 2015) the process of cell viability loss is rarely discussed.
The relationship between shear stress and red blood cell hemolysis (Paul et al., 2003, Boehning et al., 2014) or viability loss in hepatocytes (Yasuda et al., 2015) has been noted, although the process of viability loss or cell lysis was not clearly identified. Also, the effects of cell sedimentation and fluid shear stress on cell viability loss were investigated using a microchannel model to imitate the narrow channel of a catheter (Sufiandi et al., 2015). The microchannel wall shear stress was the same as with the suggested clinical condition using a 4.2 F catheter with a flow rate of 2 mL/min (Meyburg et al., 2009b). The microchannel model results showed that viability loss using a vertical syringe orientation was smaller than with a horizontal orientation, and this difference was significant under 1.3 Pa conditions (Sufiandi et al., 2015). The difference in viability losses was caused by the distribution patterns of live cells in the microchannel. The patterns determined the location of live cells in the microchannel and the intensity of shear stresses on the cells during flow in the microchannel. The cell death results indicate the need for improved practices related to cell isolation (Enosawa, 2017), transportation, and storage (Fukuoka et al., 2017) before injection for transplantation.

To understand the distribution, location, and movement of cells during flow in the microchannel, 1.3 Pa conditions were applied with the syringe in the vertical orientation, corresponding to low viability loss. This was compared with a horizontal syringe orientation (Sufiandi et al., 2017). The results showed the effects of the syringe orientation on the distribution of live cells at the upstream positions of the microchannel. The live cell distribution using a horizontal syringe orientation was concentrated at the bottom of the microchannel. In contrast, the distribution using a vertical syringe orientation was concentrated at the center of the microchannel. Cell viability loss was greatest at the bottom of the microchannel, as seen from the live cell distributions upstream and downstream.

The live hepatocytes flowed either by rolling or sliding along the bottom of microchannel, which is the primary location of viability loss. After knowing this location, it is next necessary to determine the process that causes cell viability loss. Movement along the platform was considered the main cause of cell death, and fluid shear stress was considered less likely to affect cell viability. Previous observation also found significant cells shrinkage, but very little swelling in dead cells, as measured between the upstream and downstream locations. Based on the primary location of viability loss, and the observation of significant cell shrinkage in the microchannel, the process of cell death may, in fact, be correlated with cell deformation.

To determine the primary cause of hepatocyte viability loss during the cell injection process, we studied how viability loss occurred during movement on a solid platform, and how these cells deformed when exposed to a shear force. This experiment differed from previous methods that used cells fixed in a static condition and then exposed to either a local force, as with atomic force microscopy and magnetic twisting cytometry, or a force across the entire cell, as with micropipette aspiration and optical trapping (Bao and Surresh, 2003). These methods do not test the simultaneous mechanical stressing of a population of cells under static conditions, as with shear flow and substrate stretching methods (Bao and Surresh, 2003). Our setup used cells exposed to linear shear in a controlled gap, and the condition of the cells was monitored using a microscope.

To understand the process of viability loss under similar conditions, with the same primary location in the microchannel model, this study observed the effects of direct cell contact while moving on a solid platform on cell viability, and measured the cell deformation that preceded viability loss, either via cell shrinkage or expansion. This study modeled cell movement on a microchannel platform using live hepatocytes exposed to linear shear in a controlled gap, and the condition of the cells and their deformations were incrementally observed. Two conditions, tight (25 µm) or loose (> 25 µm) gaps were employed to determine the cell deformation and simulate the various cell friction conditions caused by a solid platform.

2. Materials and methods

2.1. Sample preparation

Male Sprague-Dawley rats (specific pathogen-free) were purchased from Sankyo Labo Service., Co. Inc. (Tokyo, Japan). The animals were given standard rat chow and water ad libitum until used for isolation. Hepatocyte isolation was performed as in previous works (Hsu et al., 2013; Sufiandi et al., 2015; Yasuda et al., 2015; Sufiandi et al., 2017).

Before the experiment, the sample was stained using a dose of 20 µL propidium iodide for 1×10^6 cells. Propidium iodide is an impermeable material that is used to assess cell membrane integrity. When cells lose their membrane integrity, propidium iodide can pass through the ruptured membrane into the cytoplasm, and propidium iodide fluorescence is excited by a reaction with DNA. Then, the sample was incubated at room temperature in the range of 20°C to 26°C in the dark for 30 minutes. The samples were stored in a cold box with a temperature range of 4°C to 10°C during the experiments.
The animal experiments followed the guidelines for The Care and Use of Laboratory Animals from the National Research Institute for Child Health and Development. The experimental procedures were approved by the Institutional Animal Ethics Committee of the National Center for Child Health and Development, Setagaya, Tokyo 157-8534, Japan (Reference No. 2000-001).

2.2. Linear shear apparatus

The linear shear apparatus consisted of a base plate, top plate, linear actuator, and function generator. The base plate was a micro slide glass 76 mm × 52 mm × 1.3 mm in size (S9213, Matsunami, Osaka, Japan). The top plate was a microslide glass with dimensions 76 mm × 26 mm × 1.0 mm (S1111, Matsunami, Osaka, Japan). The separation between the plates was maintained using a thickness gauge for Group I (Nagai Gauge Co. Ltd, Osaka, Japan), and by the sample-controlled volume with Group II.

The volume of the simple shear chamber was determined from the channel width \( w \), length \( l \) and height \( h \), as shown in Table 1 (Sample volume). In the group I condition, the top plate was supported using thickness gauges with a contact width of 5 mm for each side. However, in group II, the top plate was supported by the controlled volume of the sample fluid. Therefore, the channel width for group I is narrower than with group II. The goal with Group I was to have a fixed channel height using the thickness gauges. However, the goal with Group II goal was to allow for friction-free movement of the top plate, with the same experimental shear rate and top plate displacement for the tight and loose conditions.

The parallel plates in conditions A and B of Group I were set to a separation of 25 \( \mu \)m and 30 \( \mu \)m, respectively, using thickness gauge attached to the bottom plate with vinyl tape, as illustrated in Fig. 1. The parallel plate separation in conditions C and D of Group II was set by the sample-controlled volume. The volume was calculated as the surface area of the top plate (76 mm × 26 mm) times the parallel plate separation. The separation in conditions C and D were 25 \( \mu \)m and 50 \( \mu \)m, respectively. Vinyl tape (477, 3M, St. Paul, MN, USA) was used as the guide with the top plate, as well as the fluid separator using the controlled volume. The sample volume for conditions A, B, C, and D were 30.4 mm\(^3\), 36.4 mm\(^3\), 49.4 mm\(^3\) and 98.8 mm\(^3\), respectively. [For the purposes of unit conversion when pipetting, 1 mm\(^3\) is equal to 1 \( \mu \)L, with rounded values shown in Table 1.]

The top plate was driven using a linear DC motor/actuator (LA002, Seiko, Tokyo, Japan). The actuator had an input voltage of 3 V, a current of 80 mA, and an output of 1.5 N at a velocity of 10 mm/s. A function generator (SG-4105, Iwatsu, Tokyo, Japan) was used to control the actuator. Square wave patterns, with voltages in the range of 2.8 to 4.75 volts and frequencies of 2 Hz or 3.35 Hz were used, as shown in Table 1. The linear motor drove the top plate and produced the simple shear conditions. The displacement output of the top plate for conditions A and B of Group I was recorded using a microscope and a high speed camera (NAC Memrecam GX-1 Monochrome 2 GB) with frame rate of 1000 fps. The image data were then measured using Fiji ImageJ for living science with a M Track J plug-in. The measurement results for the top plate displacement over one cycle are shown in Fig. 2

![Fig. 1 Simple shear apparatus. The experimental method to observe the process of viability loss and volume regulation using a parallel plate separation of 1.0, 1.2, or 2.0 cell diameters.](image)

The ideal shear rate \( \dot{\gamma} \) was calculated using Eq. 1, \( v \) indicated the top plate velocity, and \( h \) indicated the channel height. Therefore, the ideal shear rate \( \dot{\gamma} \) value for conditions A, C, and D was \( \dot{\gamma} = 316 \) 1/s, and in condition B was \( \dot{\gamma} = 263 \) 1/s. Representative values to calculate the shear rate employed the cell velocities measured using the high-speed camera, \( v_{cell} = 2 \) mm/s for condition A and \( v_{cell} = 1.5 \) mm/s for condition B. The shear rate \( \dot{\gamma} \) was calculated using Eq. 2.
$v_{cell}$ indicated the cell velocity and $d_{cell}$ indicated the number of cell diameters. The top plate velocities for condition C and D were controlled to maintain the same shear rate and top plate displacement. The shear rate for conditions C and D were calculated from the measured top plate velocities, $v_{plate}$, respectively, as $9.7 \text{ mm/s}$ and $19.4 \text{ mm/s}$, with the channel height $h$ as shown in Eq. 3. Representative values for both condition are shown in Table 1.

$$\dot{\gamma}_{\text{ideal}} = \frac{v}{h}$$

(1)
For condition A and B \( \dot{\gamma} = \dot{\gamma}_{cells} \)

\[
\dot{\gamma}_{cells} = \frac{v_{cell}}{d_{cell}} 
\]

(2)

For condition C and D \( \dot{\gamma} = \dot{\gamma}_{plate} \)

\[
\dot{\gamma}_{plate} = \frac{v_{plate}}{h} 
\]

(3)

The shear stress \( \tau \) was calculated using Eq. 4, with the shear rate \( \dot{\gamma} \) values for the experimental conditions shown in Table 1, assuming that William’s Medium E is a Newtonian fluid.

\[
\tau = \mu \cdot \dot{\gamma} 
\]

(4)

The Reyonld’s number \( Re \) was calculated using Eq. 5, where \( \rho \) is the density of Williams E Medium \((\rho = 1007 \text{ kg/m}^3)\), \( v \) is top plate velocity in the actuator outputs of Table 1, \( d_h \) is hydrodynamic diameter of the channel, and \( \mu \) is viscosity of Williams E Medium \( \mu = 1.325 \text{ mPa.s} \). The results of this calculation are shown in Table 1.

\[
Re = \frac{\rho \cdot v \cdot d_h}{\mu} 
\]

(5)

The hydrodynamic diameter \( d_h \) was calculated using Eq. 6

\[
d_h = \frac{2 \cdot w \cdot h}{w + h} 
\]

(6)

The Reyonld’s number \( Re_{cell} \) was calculated using Eq. 7 with \( d_{cell} \) indicating the cell diameter and \( v_{cell} \) indicating the cells velocities.

\[
Re_{cell} = \frac{\rho \cdot v_{cell} \cdot d_{cell}}{\mu} 
\]

(7)

The channel fluid flow parameters in Table 1 possess low Reynold’s numbers, and indicate that oscillating linear shear conditions can occur. At high Reynold’s numbers, Leider’s squeezing flow (Leider and Bird 1974a; Leider and Bird 1974b) may occur instead.

The simple shear apparatus was attached to the observation stage of an inverted microscope (Eclipse TE2000-U, Nikon, Tokyo, Japan). The microscope used 4\( \times \) objective lens magnification (Plan Fluor 4X, Nikon, Tokyo, Japan) and a filter for the propidium iodide (G-2A, Nikon, Tokyo, Japan). The light sources were a 100 Watt mercury lamp and a 100 Watt halogen lamp.

At \( t = 0 \text{ s} \), the cells were observed as an initial condition, without any shear or periodic shear stress. Then, the cells were exposed to shear for 2 second intervals for group I (A and B), and 5 second intervals for group II (C and D). Images showing both the fluorescence and brightfield microscopy data were recorded using a high-resolution camera (ILCE-QX1, Sony, Tokyo, Japan). The camera resolution was 5456 \( \times \) 3632 pixels, with an exposure time of 1/160 s. The camera viewing area was 6.76 mm \( \times \) 4.5 mm, which represented 1.54\% of the total surface area. The fluorescence and brightfield images were merged using ImageJ (National Institutes of Health, Bethesda, Maryland, USA). Then, the number of cells that excluded propidium iodide were manually counted using the ImageJ cell counter, which distinguishes between viable and dead cells.

The ratio of live cells, which is the number of viable cells compared with the initial value, was used when presenting the data. The cell loss ratio is the difference between the live cell ratio of the observed condition and live cell ratio of the reference condition. The data are reported for each 10-second time interval, and the observation range covered 0 to 40 seconds. The error bars in the plots represent the standard errors. Student-t tests were performed, and a single or double asterisk indicates \( p < 0.05 \) and \( p < 0.005 \), respectively.

To understand the process of cell viability loss, the areas of live and dead cells were compared. Measurements were taken at 10 second intervals in the tight gap (25 \( \mu \text{m at 388 1/s} \) of condition C, and the loose gap (50 \( \mu \text{m at 388 1/s} \) of condition D, with a cell sample number of \( n = 5 \). The camera has a resolution of 5456 \( \times \) 3632 pixels, using 4\( \times \) magnification, 100 \( \mu \text{m correspond to 80.67 pixels in the image} \), and could be used to track the cell boundary during the cell area measurements. The cell areas at time \( t \) of observation \( A_t \) were measured using ImageJ, and the cell boundary was tracked manually using the trackpad, which allowed the cell area to be measured using imageJ. The cell area at the time of observation \( A_t \) were normalized to the live cell area at \( t = 0 \text{ second}, A_0 \) using Eq. (8) and shown as the deformation ratio \( \lambda \)

\[
\lambda = \frac{A_t}{A_0} 
\]

(8)

Cell deformation could be in the form of shrinkage, as indicated by \( \lambda < 1 \), or expansion, as indicated by \( \lambda > 1 \)
3. Results and discussion

3.1. Rapid loss of cells under initial shear conditions

Figure 3 shows the live cell ratio during simple shear treatment at 10 second intervals. Figure 3-Control shows condition A without any shear treatment. The results indicate no significant change in live cell ratio over the observation time. A large decrease in the number of live cells occurred under simple shearing in all experimental conditions. This was seen by comparing the live cell measurements 10 to 40 seconds after the beginning of the experiment with the initial values. The results show significant differences for all conditions, except for the live cell ratio after 10 seconds under condition D. The results indicate that shear induced cell viability loss.

Significant initial cell loss >20% occurred under conditions A, B, and C, as indicated by a comparison of the cell loss ratios with their initial values (Fig. 4). The results show that the cell loss in conditions A and B were significant at all times. An exception was the cell loss between 30 to 40 seconds under condition C. In contrast, the cell loss under condition D was not significant at any time. These results indicate that direct contact on a solid surface is destructive for cells.

The large initial cell loss may be the result of a loss of membrane integrity on the part of low-quality cells during the initial 10 seconds of shear. This loss of cell membrane integrity can be noted in the cells that became stained with propidium iodide or were no longer visible. The disappearance of a cell body indicates total membrane rupture. Significant initial cell loss only occurred in the tight gap (25 µm and 30 µm) conditions of A, B, and C, as shown Figs. 4A-C. Therefore, low-quality cells were destroyed under the tight-gap condition.

The initial viability losses over the first 10 seconds were compared (Fig. 5). The results show that condition B had the highest initial viability loss, which was significantly different than with condition D, which had a higher shear rate and a larger gap. This indicates that rapid and significant viability loss might be caused by deformation in low-quality cells during contact with the solid interface, and that the shear rate had a smaller influence on cell loss.
Low-quality cells may be the result of the preparation process before shear exposure, which include cell isolation, fluorescence staining, transportation, and storage. These processes might reduce cell quality via physical or biological factors. Cell contact with a solid surface during sample transportation is a physical factor that might cause deterioration of cell quality. Biological factors that could influence cell quality include damage during reperfusion or isolation, or the effect of hypothermic temperatures during storage (Vreugdenhil et al., 1996).

### 3.2. Threshold of cell viability loss

The threshold limit of cells viability loss occurs when the incremental cells losses that are different than for previous increments. Cell death during the 30–40 second time interval was considered the threshold limit of cell viability loss. As shown by the differences between the 10–20 second interval in condition A (Fig. 4A) compared with 10–20 and 20–30 second intervals of condition C (Fig. 4C), condition A had a lower shear rate (78 1/s) that did not exceed the cell threshold limit of cell viability loss. Conversely, the high shear rate of condition C (388 1/s) corresponded to cells that
exceeded the threshold limit of viability losses. Therefore, the tight gap of 25 µm in conditions A and C restrict cell deformation until the threshold is reached. The threshold limit of cell viability loss also occurred during the 30–40 second interval of condition B (30 µm at 61 1/s), as indicated by a higher mean value compared with the 10–20 and 0–30 second interval. The threshold limit of cell viability losses is an indication that the threshold limit of cell viability was exceeded, demonstrating irreversible cell damage.

On the other hand, the threshold limit of cell viability losses in condition D was not observed, because the cells could become freely deformed in this condition. Condition D models the viability loss in a microchannel (Sufiandi et al., 2017) with 100% more separation (50 µm) and a higher shear rate (388 1/s). The number of surviving cells in condition D decreased with increasing exposure time, as shown in Fig. 3D, with a Pearson correlation value for cell survival with exposure time $r = -0.99$. Therefore, the number of live cells were lost at a relatively constant rate during each increment, as shown in Fig. 4D. In summary, the results indicate that cell viability loss mainly was influenced by cell deformation when exposed to shear.

3.3. Delayed cell viability loss

The cell losses between 10 to 30 seconds were compared to understand the behavior prior to the threshold condition, as shown in Fig. 6. The cell loss ratio during the time period between 10 to 30 seconds (Fig. 4) reflected cellular deformation, which occurred before the threshold limit of viability loss was reached. Cell deformation might have influenced the process of viability loss because, under tight gap conditions, cell deformation was restricted by friction between the cell and the parallel plate walls. This delayed the process of cell viability loss, as indicated by a comparison between the tight gap of condition C (25 µm at 388 1/s) and the loose gap of condition D (50 µm at 388 1/s). The results show that cells exposed to shear that had space to deform (loose gap) suffered more damage than those in a tight gap. On the other hand, cell friction might have triggered cell viability loss, as indicated by a comparison between the high shear of condition C (25 µm at 388 1/s) and the low shear of condition A (25 µm at 78 1/s). Friction in a low-shear condition (A) might be higher than in a high-shear condition (C). Reducing friction by using a 5 µm larger gap may reduce cell viability losses, as indicated by a comparison between condition A (25 µm at 78 1/s) and condition B (30 µm at 61 1/s). Because friction is dependent on the contact area of surfaces, a smaller contact area will tend to reduce friction. If a cell has no contact with any surface, no friction will be generated, and viability losses may be prevented. Therefore, reducing or eliminating friction may preserve cell viability.

3.4. Deformation ratio

The cell deformation ratio $\lambda$ in the tight gap (25 µm at 388 1/s) of condition C, and loose gap (50 µm at 388 1/s) of condition D are shown in Fig. 7. The measurement of the cellular areas and calculation of cell deformation ratios, show that the cell sizes decreased (shrunk) after exposure to shear for 10 or 20 seconds post-treatment, and then increased in size (expanded) 30 to 40 seconds post-treatment. These results indicate that the process of cell viability loss is involved cell shrinkage as a response of shear on a cell body. Cell shrinkage was significant compared with the initial size, with $\lambda$ in the range of about 0.8 to 1. In the time interval of 30 to 40 seconds, the cell deformation ratio was $\lambda > 1$. Cell deformation
was significant compared with the initial size, with $\lambda$ in the range of about 1 to 1.3.

**Fig. 7** Deformation ratio of cells over a 10 second increment. A indicates the tight gap (25 $\mu$m at 388 1/s) of condition C, and B indicates the loose gap (50 $\mu$m at 388 1/s) of condition D. During the process of viability loss, the cells deformed by decreasing their size (cell shrinkage), defined as "zone 1," then the cells deformed by increasing their size (cell expansion), defined as "zone 2." The condition of cells in zone 1 was predicted to be reversible, when the expansion process in zone 2 was considered irreversible. n=5 for each increment. The error bars indicate the standard errors. * p <0.05, ** p <0.005

Cell deformation in condition C (388 1/s at 25 $\mu$m) was limited by the tight gap, as indicated by the observation 30 seconds post-treatment, which saw deformations in the range of 1 to 1.1, as compared with condition D (388 1/s at 50 $\mu$m), which had deformations in the range 1.2 to 1.3. The deformation of dead cells in condition C was also limited by the tight gap, as indicated by the continuous cell expansion between 0 and 30 seconds, with $\lambda$ about 1.5, after which the cells shrank to the range of $\lambda$ 0.9 to 1.1. On the other hand, the deformation of dead cells in condition D was in the range of 0.9 to 1.1. Cell death occurred after a loss of membrane integrity in the loose gap (50 $\mu$m), when the cell first increased in size, then shrank when not limited by friction from the parallel plate boundaries under loose gap conditions. The deformation ratios for live and dead cells under condition D were in good agreement with the deformation ratio of cells flowing on the bottom of a microchannel, observed at upstream and downstream locations (Sufiandi et al., 2017). The cell shrinkage occurred between 0 to 10 seconds (zone 1), and expansion occurred between 10 seconds to 40 seconds (zone 2), as shown in Fig. 7.

### 3.5. Process of immediate cell viability loss

The process of cell viability loss was divided into two zones, as illustrated in Fig. 7. Cell shrinkage (zone 1) occurred before expansion, and is considered safe for cells. Cell expansion, however is consider irreversible and fatal, since the cells will continue expanding until a loss of membrane integrity or ruptured occurs. The time required for cell death was less than 10 seconds, and some cells were dead within 5 seconds.

Cell shrinkage and expansion might be related to poroelasticity (Moeendarbary et al., 2013). Cell shrinkage in response to shear is an interesting finding, because it might be related to apoptosis (Maeno et al, 2000), regulated volume decreases (Okada et al., 2001), surface area regulation, (Staykova et al., 2011; Morris and Homann, 2001) or water efflux from the cells (Heo et al., 2012). However, cell shrinkage in these reports takes place over a time on the order of seconds, while apoptosis (Maeno et al, 2000) and surface area regulation (Staykova et al., 2011) require times on the order of hours. The process of cell shrinkage seen here might not be influenced by regulated volume decreases (Holtzclaw et al., 2010; Okada et al., 2001), which involves K$^+$ and Cl$^-$ ion channels over a timescale of minutes, but may occur via aquaporins (Heo et al., 2012), which have a shorter timescale, and are considered fundamental to mechanically-induced cell death (Galluzzi et al., 2015). Therefore, the shrinkage process might not be related with apoptosis, surface area regulation or regulated volume decreases. Cell deformation might be related to F-actin changes in the cytoskeleton, which influences...
cell shape (Ohashi et al., 2008; Ujihara et al., 2010), and the mechanism of cell shrinkage and expansion might be related to depolymerization or excess polymerization of the F-actin cytoskeleton (Moeendarbary et al., 2013).

3.6. Morphology of immediate cell viability loss

Images using the tight gap (388 1/s at 25 µm) of condition C, or loose gap (388 1/s at 50 µm) of condition D, were observed and tracked to understand the process of viability loss. Cell death was considered to have occurred when a cell became stained by the membrane impermeable marker, propidium iodide. The loss of cell membrane integrity was tracked over 5 second intervals. As an example, a typical cluster of cells in a loose gap (50 µm) under condition D is shown in (Fig. 8). The initial condition of cells in a cluster is shown in Fig. 8A. Some cells in the cluster were healthy, but after being loaded for 5 seconds, became stained, as shown in Fig. 8B. The fluorescence intensity in Fig. 8C increased after 5 seconds of shear exposure. The surfaces of live cells decreased in size, as seen in Figs. 8B and C. Thus, the results show that under loose gap conditions (50 µm) cell viability loss occurs quickly, in less than 5 seconds. The effect of cell deformation under loose gap conditions on cell viability loss is in good agreement with the results of condition D, in which a loose gap was more destructive to cells than a tight gap (Fig. 6).

![Fig. 8](image)

Cell morphology of viability loss using the loose gap (388 1/s at 50 µm) of condition D.
The increment between images is 5 seconds. The arrows in Fig. 8 A, B, and C show intact cells, stained cells, and increased stain intensity, respectively. The double arrowheads indicate the direction of the shear for all subfigures.

3.7. Morphology during delayed viability loss

The delayed process of viability loss for a typical cell in the tight gap (388 1/s at 25 µm) of condition C is illustrated by the observation of two cells in the cluster shown in Fig. 9. The propidium iodide fluorescence intensity of the stained cells, as indicated by the filled arrow, increased during each 5-second interval. This result shows the gradual process of cell viability loss. Intact cells in the cluster are marked by the open arrow in Figs. 9A–E. The threshold limit of cell viability is shown in Figs. 9E–F. The threshold limit of cell viability is when the cells begin to lose their membrane integrity. Viability loss might occur when a cell exceeded this threshold limit, and then lost its membrane integrity, as indicated by the propidium iodide stain. Cells stained by propidium iodide are shown in Figs. 9F–H. This cellular morphology observation reflects delayed cell viability loss, as seen in Fig. 4C. The delayed cell viability loss in the tight gap (25 µm) of condition C might be an effect of the space limitation of the parallel-plate gap, which restricted cells from becoming freely deformed. The effect of cell deformation in a tight gap on delayed cell viability loss was seen in condition C (Fig. 6).

3.8. Cell membrane ruptured

3.8.1. Single cell observation

Extended observations of viability loss leading to cell membrane rupture in the tight gap (25 µm at 388 1/s) of condition C are shown in Fig. 10. Two live cells in the cluster were tracked in 5-second time intervals. The purpose was to understand how cell deformation occurred under extended shear prior to cell rupture. The cell cluster was tracked from 5 to 60 seconds. This images reflect 10 second intervals (Fig. 10A). After 60 seconds, one cell separated from the cluster and was observed from 65 to 260 seconds, until the cell membrane ruptured (Fig. 10B).

3.8.2. Deformation ratio

Approximately 52 images were acquired to observe the response of a cell to shear under tight-gap (25 µm) conditions. The cells were analyzed by measuring their areas and calculating the equivalent diameters. Each cell was measured from the initial condition until it ruptured. The measurements ended with the last cells still intact.
Fig. 9  **Cell morphology of delayed cell viability loss in the tight gap (388 1/s at 25 µm) of condition C.**
The interval between images is 5 seconds. Open arrows indicate intact cells, and filled arrows indicate stained cells. The double arrowheads indicated direction of the shear for all subfigures.

Fig. 10  **Cell morphology of two cells in a cluster prior to cell rupture.** A. Two cells in a cluster. ● indicate a separated cell from the cluster; B. Single cell monitored until rupture ○; C. Deformation ratio of the observation. The double arrowheads indicate the direction of the shear for all subfigures.

after 245 seconds. The deformation ratio $\lambda$ is a comparison of the cell area to its initial value. The deformation ratios of the cells is presented in Fig. 10C. The results show a contraction of the deformation ratio, in the range of 0.9 to 1.4. Thus, cells can contract up to 40% before rupture.

3.9. **Shear rate and shear stress in clinical practice, microchannel model, and simple shear condition**

The safe hepatocyte transplantation infusion conditions suggested for clinical practice include a 4.2 F catheter with a flow rate of 1 mL/min (Meyburg et al., 2009b). As comparison with 9.6F, 6.6F, 5F, 18G and 4.2F catheter sizes used in clinical conditions with internal diameters of, respectively, 1.6 mm, 1.1 mm, 0.95 mm, 0.84 mm and 0.7 mm. Using a flow rate 1 mL/min (Sufiandi et al., 2015) these have velocities, shear rates, and shear stress 25 µm from catheter wall, respectively, (2.0 mm/s, 80 1/s, 0.1 Pa), (6.2 mm/s, 248 1/s, 0.3 Pa), (9.6 mm/s, 384 1/s, 0.5 Pa), (13.9 mm/s, 556 1/s, 0.7 Pa) and (23.9 mm/s, 1.3 1/s, 956Pa). On the other hand, the velocity, shear rate, and shear stress in the microchannel experiment at 25 µm from the bottom of the microchannel was (8.8 mm/s, 352 1/s, 0.5 Pa) (Sufiandi et al., 2015; Sufiandi et al., 2017). The velocities, shear rate, and shear stress in this study was (2.0 mm/s, 78 1/s, 0.1 Pa) (1.5 mm/s, 61 1/s, 0.1 Pa) (9.7 mm/s, 388 1/s, 0.52 Pa) (9.7 mm/s, 388 1/s, 0.52 Pa), respectively, for conditions A, B, C, and D. Therefore, the
shear rate and shear stress in this study could represent clinical conditions experienced during hepatocyte transplantation.

4. Conclusion

This study found that the direct contact of a hepatocyte with a solid surface is deleterious to the cell, and cell deformation leading to viability loss is caused by cell shrinkage and expansion. The reduction in cell size, up to a certain limit, is considered reversible and not fatal. However, once the limit of cell shrinkage is exceeded, the cell may continue to deform via expansion until loss of membrane integrity or rupture occurs in an irreversible process. Therefore, cells may only be safely deformed up to the cell shrinkage limit. Cell expansion can be prevented by reducing the height of the channel. However, this does not ameliorate deformation due to friction caused by the direct contact of a hepatocyte with a solid surface. In conclusion, preventing the direct contact of a cell and reducing friction is necessary to improve cell viability associated with injection during hepatocyte transplantation.

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