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RESEARCH ARTICLE

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Protection of human induced pluripotent stem cells against shear stress in suspension culture by Bingham plastic fluid

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Abstract

Suspension culture is an important method used in the industrial preparation of pluripotent stem cells (PSCs), for regenerative therapy and drug screening. Generally, a suspension culture requires agitation to keep PSC aggregates suspended and to promote mass transfer, but agitation also causes cell damage. In this study, we investigated the use of a Bingham plastic fluid, supplemented with a polysaccharide-based polymer, to preserve PSCs from cell damage in suspension culture. Rheometric analysis showed that the culture medium gained yield stress and became a Bingham plastic fluid, after supplementation with the polymer FP003. A growth/death analysis revealed that 2 days of aggregate formation and 2 days of suspension in the Bingham plastic medium improved cell growth and prevented cell death. After the initial aggregation step, whereas strong agitation (120 rpm) of a conventional culture medium resulted in massive cell death, in the Bingham plastic fluid we obtained the same growth as the normal culture with optimal agitation (90 rpm). This indicates that Bingham plastic fluid protected cells from shear stress in suspension culture and could be used to enhance their robustness when developing a large-scale.

KEYWORDS

Bingham plastic fluid, pluripotent stem cells, shear stress, suspension culture

1 | INTRODUCTION

Pluripotent stem cells (PSCs) are one of the most promising cell sources for regenerative therapy[1-3] and drug screening[3,4] owing to their special characteristics, such as extensive growth and the potential to differentiate into any type of body cell. For the industrial production of PSCs, stable mass production systems are necessary to establish a cell bank and to regenerate large organs such as the liver, heart, and pancreas.[5] Despite many efforts, establishing industrial systems that can meet the basic mass production requirement of $>10^{10}$ cells per batch[5-7] remains a challenge.

PSCs may be cultured in suspension, and such systems have been much studied.[8-11] Suspension culture does not require a surface for adhesion, so culture vessels can be simpler than those used in conventional adhesion culture. For the mass production of PSCs, various culture systems and vessel designs have been tested. However, there are still barriers to realizing a stable suspension culture of PSCs. For example, agitation is required to maintain the flotation and

distribution of PSC aggregates in the vessel. In conventional suspension culture, PSC aggregates settle and gather in the middle of the bottom of the vessel, which increases the collision and fusion of aggregates, the outcome of which is a reduction in cell yield. However, PSCs are also sensitive to shear stress; therefore, strong agitation causes massive cell death. These two competing factors limit the operating conditions and prevent the establishment of stable cultures. There have been many attempts to address this issue,[11-15] to limited effect, and the problem persists.

To address the problem of shear stress, utilizing some polymers to modify the character of medium is one option. Polysaccharides[16-18] and poloxamer[16,19] were added to modify the cell viability in suspension culture. These polymers increased the viscosity and encapsulate cells to protect from hydrodynamic damage.[16] High viscosity medium can maintain cell viability but increase the viscosity caused a difficult handling such as separation, medium change and mixture. To improve this problem, the use of non-Newtonian fluids (which change viscosity with stress) is one of the other promising approaches. Otsuji and colleagues[15] established a method of suspension culture for PSC aggregates within a non-Newtonian medium containing gellan gum. In their report, the medium successfully kept PSC aggregates floating without agitation. However, the scale was small (250 ml) and oxygen was supplied through the oxygen-permeable membrane of the culture bag. Unfortunately, active oxygenation and mixing (via agitation) are essential for further scaling up (>1 L) because of the need to transport nutrients and oxygen to the cells, and the effect of using a non-Newtonian medium on cells in dynamic flow is not well known.

In this study, we investigated the effect of a non-Newtonian medium on cell behaviors, aggregate formation, growth, and death in suspension cultures, with agitation. In the experiments, we attempted a small-scale suspension culture of human induced pluripotent stem cells (hiPSCs). They were cultured in a polysaccharide-based polymer mix, which made the culture medium a non-Newtonian fluid. After four-days of cultivation under suspension conditions, we measured the cell growth and the number of dead cells to understand how non-Newtonian fluid protects cells from shear stress.

2 | MATERIALS AND METHODS

2.1 | Maintenance of hiPSCs

HiPSC line 15M63 was purchased from Kyoto University. Cells were seeded on a tissue-culture-treated 100 mm dish (Iwaki), coated with vitronectin fragments (VTN-N, Thermo Fisher Scientific, Waltham, MA) and maintained in StemFit AK02N (Ajinomoto, Tokyo, Japan). The culture medium was replaced every 24 hr. Accutase (Innovative Cell Technologies, San Diego, CA) was used to passage 15M63. The number of passages was <50 for experimental use.

A second hiPSC line, TkDN4-M, was provided by Stem Cell Bank, Center for Stem Cell Biology and Regenerative Medicine, The University of Tokyo. They were seeded on a vitronectin fragment-coated dish and maintained in Essential 8 medium (Thermo Fisher Scientific) containing Gibco^{R} Antibiotic-Antimycotic (Thermo Fisher Scientific). The culture medium was replaced every 24 hr. Calcium- and magnesium-free phosphate buffered saline (PBS, FUJIFILM Wako Pure Chemical, Osaka, Japan) containing 0.5 mM ethylenediaminetetraacetic acid (EDTA, Dojindo, Kumamoto, Japan) was used to passage TkDN-4M. The number of passages was <50 for experimental use.

2.2 | Preparation of non-Newtonian culture medium and rheometric analysis

Non-Newtonian culture medium was prepared using the FCeM advance preparation kit (Nissan Chemical, Tokyo, Japan) according to the guidance from the company. Briefly, 10 ml of FP003 polymer solution was added to 200 ml of culture medium. The prepared mixture was added through the cell strainer into the rest of the basal medium. After preparation, the non-Newtonian medium was utilized for suspension culture. To understand the rheology of the medium, shear stress at various shear rates (1–500 s^{-1}) was measured by a rheometer (MCR301, Anton-Paar, Graz, Austria) at 25°C.

2.3 | Evaluation of aggregate distribution in non-Newtonian fluids

HiPSC aggregates were obtained by suspension culture, using a 90 rpm orbital shaker (CS-LR, Taitec, Saitama, Japan) for 2 days, as mentioned below. Collected aggregates were stained with Coomassie Brilliant Blue (Quick-CBB Plus, FUJIFILM Wako Pure Chemical). Stained aggregates were suspended in 4 ml of phenol-red-free Dulbecco's modified Eagle medium (FUJIFILM Wako Pure Chemical) with/without FCeM, in a 6-well plate. To observe the distribution pattern of hiPSC aggregates, the aggregate movement was captured by a high-speed camera during orbital shaking (Q-cam, nac image technology, Tokyo, Japan).

2.4 | Suspension culture of hiPSCs

HiPSCs were dissociated into single cells using a dissociating solution (Accutase or EDTA) and collected in culture medium with 10 μM Y-27632 (FUJIFILM Wako Pure Chemical). Following a cell counting using the trypan blue exclusion test, 8×10^5 cells were seeded in 4 ml of culture medium (final inoculum cell density was 2×10^5 cells/ml) with 10 μM Y-27632 on low-cell-attachment-treated 6-well plates (Thermo Fisher Scientific). Seeded cells were incubated at 37°C and 5% CO_2 and agitated at 90 rpm using an orbital shaker for 2 days, to form aggregates. After 2 days the culture medium was replaced with Y-27632-free medium with/without FP003, and the agitation rate was changed, if required. The medium was renewed again on day 3 and cells were collected for analysis on day 4. Medium samples were collected on days 2, 3, and 4 for the measurement of lactate dehydrogenase (LDH).

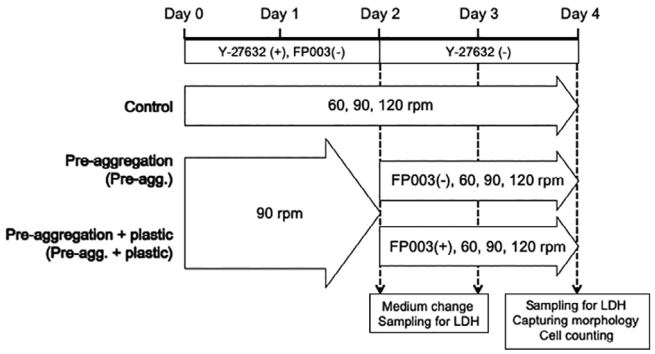

FIGURE 1 Schematic diagram of the experimental procedure

The experiment was conducted with a total of 9 conditions, at three different agitation rates (60, 90, and 120 rpm) and two different schedules of agitation rate and culture medium (Figure 1). As mentioned above, the initial aggregation process was performed by shaking at 90 rpm for 2 days in the culture medium with Y-27632, without FP003. The other group was cultured at various shaking rates (60, 90, and 120 rpm) immediately after seeding. After 2 days of initial aggregation, the culture medium was switched into two different culture media (with/without FP003) and cultured for more than 2 days under various shaking conditions (60, 90, and 120 rpm).

2.5 | Measurement of cell growth and death

After 4 days of culturing in suspension, cells were collected and dissociated into single cells by incubating in TrypLE Express (Thermo Fisher Scientific) for 5 min. Following pipetting for dissociation, the viable cell number was counted by the trypan blue exclusion test, with an eosinophil counter.

The cumulative number of dead cells was estimated by measuring the LDH released from cells in the collected medium samples. Samples were collected at each change of medium, on days 2 and 3, and after harvest. LDH concentrations were measured by LDH-Cytotoxic Test Wako (FUJIFILM Wako Pure Chemical) and a fluorescence microplate reader (Arvo-SX, PerkinElmer, Waltham, MA) according to the manufacturer's instructions. A standard curve was obtained by measuring the amount of LDH from various densities of hiPSC suspension (1×10,^{3} 1×10,^{4} 1×10,^{5} 2×10,^{5} and 3×10^5 cells/ml), treated with phosphate-buffered saline containing 0.2% Tween20.

2.6 | Histological analysis of aggregates after suspension culture

The hiPSC aggregates were fixed with 4% paraformaldehyde solution (FUJIFILM Wako Pure Chemical, Japan) in PBS for 30 min at room temperature. Afterwards, the fixed aggregates were washed with PBS and incubated with 30% PBS buffered sucrose solution, at 4°C overnight, followed by embedding with optimal cutting temperature (OCT) compound (Sakura Finetek, Tokyo, Japan) in a cryomold at −20°C, until hard. The thin cross-sections of aggregates were obtained using a CM3050 cryostat (Leica, Wetzlar, Germany) and mounted onto glass slides for hematoxylin–eosin (HE) staining.

2.7 | Flow cytometry analysis

To obtain single cells, the hiPSC aggregates were dissociated by Accumax Cell Aggregate Dissociation Medium (Invitrogen) and passed through a 40 μl cell strainer (Corning). Then, 10^6 cells were fixed with 4% paraformaldehyde solution (FUJIFILM Wako Pure Chemical) in PBS for 30 min at room temperature, followed by permeabilization with 1% Triton X (Sigma–Aldrich) in PBS for another 30 min, at room temperature. After washing with HBSS, the hiPSCs were incubated for 1 hr with monoclonal mouse IgG2a Anti-Human Oct-4A Alexa Fluor 488-conjugated antibody (R&D Systems). The control group was incubated with anti-mouse IgG isotype control (R&D Systems) at room temperature. The stained cells were washed with HBSS and the flow cytometry analysis performed by Epics XL (Beckman Coulter, CA). The data were analyzed using Expo32 ADC analysis software (Beckman Coulter).

2.8 | Statistical analysis

Quantitative data for statistical analyses were obtained from six samples in two different independent experiments. For the significance test, the Student's *t* test was performed by using Excel.

3 | RESULTS

3.1 | Characterization of non-Newtonian culture medium

According to rheological analysis of the basal medium with/without FP003, without FP003 the medium showed typical Newtonian fluid character, whereas with FP003 it had higher viscosity and showed Bingham plastic fluid behavior, with a yield force (open plots in Figure 2). The yield force indicates the minimum force for flow, which means that sedimentation does not occur when the gravitational force on the cells is < the yield force.[20,21] Culture media gain yield force by their content of protein, such as albumin, and the addition of FP003 enhanced the yield force and increased viscosity (Figure 2).

The plots of viscosity as a function of shear rate showed that FP003 increased viscosity in lower shear rate (Figure 3). This trend was more prominent in the medium with the supplement (Figure 3b). In case of the medium with the supplement, the medium without FP003 lost high viscosity immediately after increasing shear rate and showed similar viscosity to that at higher shear rate (>100 s^{-1}). Meanwhile, the medium with FP003 sustained higher viscosity at low shear rate (<20 s^{-1}) than that at higher shear rate (>100 s^{-1}). At the higher

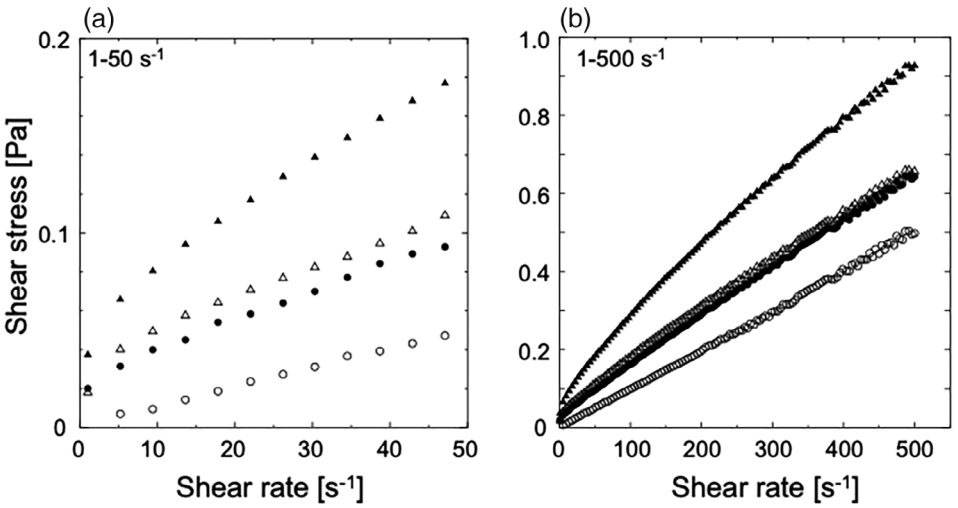

FIGURE 2 Rheological analyses of basal medium with/without FP003 and with/without the supplement in different ranges, (a) 1–50 s^{-1} and (b) 1–500 s^{-1}. Black circles indicate the rheology of the basal medium with FP003, and white circles indicate that of the basal medium without FP003. The black triangles indicate the rheology of the complete medium including growth factor supplements and FP003, and the white triangles indicate that of the complete medium including growth factor supplements but without FP003

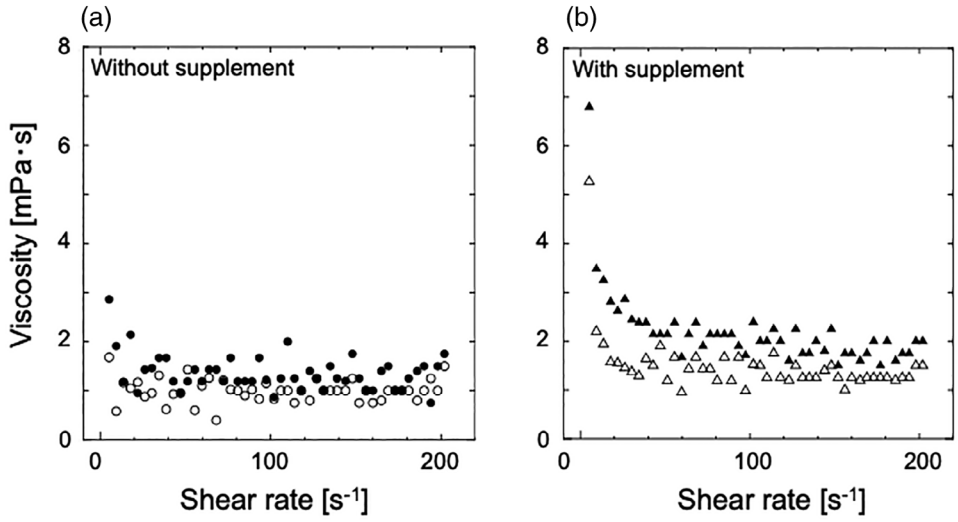

FIGURE 3 Profile of viscosity as a function of shear rate of medium (a) without supplement and (b) with supplement. The range of shear rate was from 1 to 200 s^{-1}. Black symbols indicate containing FP003 and white symbols indicate without FP003

shear rate (>100 s^{-1}), viscosity was not remarkably changed comparing with that at lower shear rate and showed from 0.75 to 2.4 mPa·s.

Visualization of the distribution of stained cell aggregates, in orbital shaking suspension, showed that the non-Newtonian fluid promoted cell distribution at lower shaking rates. In a conventional medium, aggregates settled down and gathered into the center-bottom of the vessels. This is because of medium flow due to agitation, known as Einstein's tea-leaf paradox.[22] On the other hand, aggregates in the non-Newtonian medium were kept floating and distributed (Figure S1).

3.2 | Cell morphologies during and after suspension culture with/without non-Newtonian fluid

According to the morphological observation of aggregates after the initial 2 days post seeding, at various agitation rates, those forming under slow (60 rpm) or fast (120 rpm) agitation exhibited difficulties (Figure 4). With slow agitation, cells over-aggregated, forming large aggregates which caused low growth due to contact inhibition and the limitation of nutrient supply. With strong agitation, there was cell damage which led to failure of aggregate formation. Therefore, we used a 2 day process of initial aggregation, by shaking at 90 rpm, prior to changing and evaluating the effect of agitation rate. In addition, our preliminary experiment showed that the presence of FP003 at initial aggregation decreased the number of aggregates to one-third of that in conventional medium (Figure S2). Therefore, we decided to evaluate FP003 only after two days of aggregate formation.

Morphological observation suggested that culturing in a non-Newtonian fluid improved the tolerance of aggregates to the problems of being in a suspension culture. Even after 2 days of aggregate formation within a Newtonian culture medium, with 90 rpm orbital shaking, the agitation was inadequate and caused failure of the culture. With slow agitation, aggregates fused to form large masses, but fast agitation distorted and broke down the aggregates. With the use of a non-Newtonian fluid, the morphology of the aggregates improved, and were as good as a Newtonian culture medium under optimal agitation. These results showed that the non-Newtonian

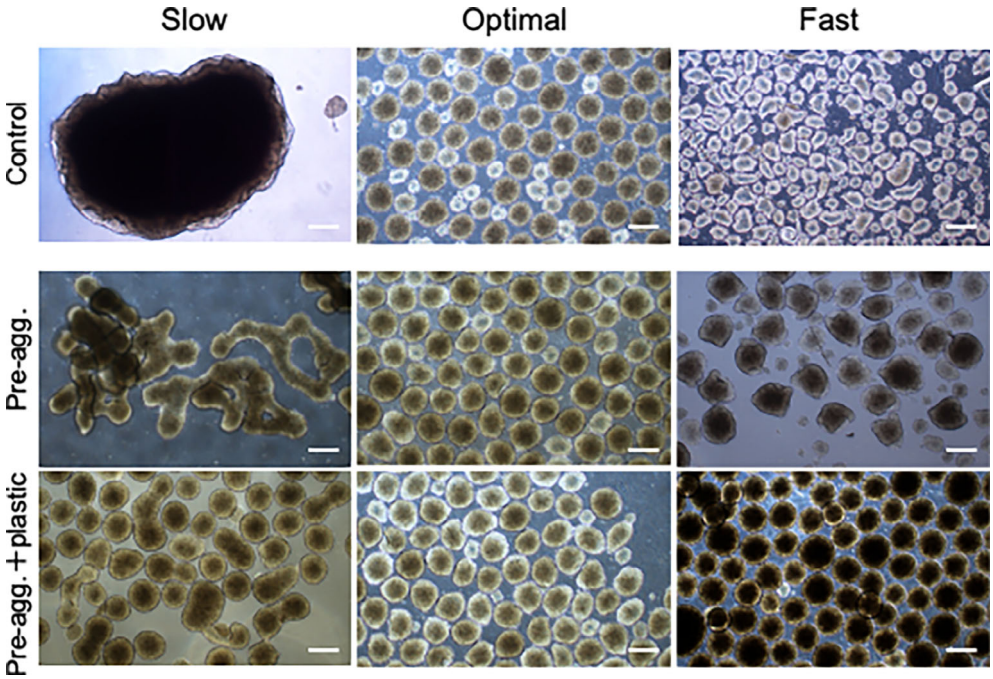

FIGURE 4 Morphological images of hiPSC aggregates after 4 days of culture at various shaking rates, with different culture media: plastic = Bingham plastic fluid; control = Newtonian fluid; pre-agg. = pre-aggregation. Scale bars indicate 400 μm

culture medium protected hiPSCs from some problems that are typical of suspension cultures, such as fusion of aggregates and flow damage.

The HE staining of cross sections of aggregates showed that cells survived in the aggregates in both media, with or without FP003, and with insignificant difference in morphology (Figure S3). This indicates that FP003 was nontoxic and did not affect the viability or morphology of cells. In both conditions, inner cells also survived without necrosis, which suggests that the non-Newtonian medium did not prevent oxygen supply in a laboratory-scale suspension culture.

3.3 | Cell growth, death, and pluripotency during suspension culture

Cell growth analyses showed that an inadequate agitation rate in Newtonian culture medium resulted in low cell growth due to excessive aggregation or shear stress (Figure 5). In particular, the number of viable cells decreased after fast shaking. Two days of pre-aggregation in optimal conditions improved cell growth, but a subsequent non-optimal shaking rate still decreased cell viability compared to data from optimal shaking. According to further analyses, the non-Newtonian culture medium improved cell growth at each agitation rate, from inappropriate to optimal shaking conditions. This suggests that the non-Newtonian culture medium improved the robustness of cells to shear stress from agitation.

According to cell death measurement, based on leaked LDH, more deaths occurred at a higher agitation rate, due to higher shear stress (Figure 6). Initial aggregate formation reduced cell death, except in the case of 60 rpm, due to the improvement of cell growth during initial aggregation. In addition, non-Newtonian fluid decreased cell death at every shaking speed. In summary, the use of initial

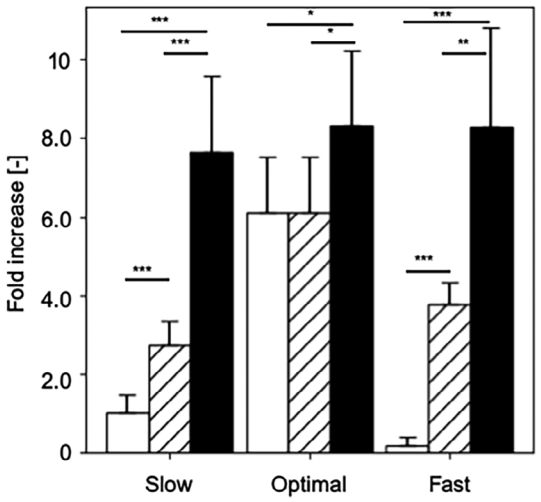

FIGURE 5 Fold increase in initial cell density (8×10^5 cells/4 ml) after 4 days of culture at various shaking rates. The white bar indicates the result of the control group, the hatched bar indicates that of the pre-aggregation group, and the black bar indicates that of the plastic medium and pre-aggregation group. The average value was derived from 6 samples in 2 independent experiments. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

aggregation and non-Newtonian culture medium minimized cell death and increased cellular growth, improving the robustness to shaking.

According to flow cytometry analysis of OCT3/4, the hiPSCs were able to sustain OCT3/4 expression (Figure S4), suggesting that the FP003 polymer did not inhibit pluripotency during culture.

To evaluate the difference of the effect of Bingham plastic medium on cell growth and death among the cell lines, we performed the same experiment of other cell line, TkDN-4M. In case of

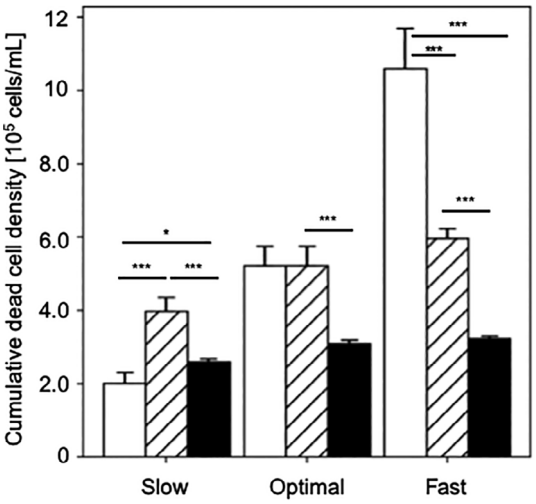

FIGURE 6 Cumulative dead cell density calculated from the amount of lactate dehydrogenase leaked from damaged and dead cells. The white bar indicates the result of the control group, the hatched bar indicates that of the pre-aggregation group, and the black bar indicates that of the plastic medium and pre-aggregation group. The average value was derived from 6 samples in 2 independent experiments. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

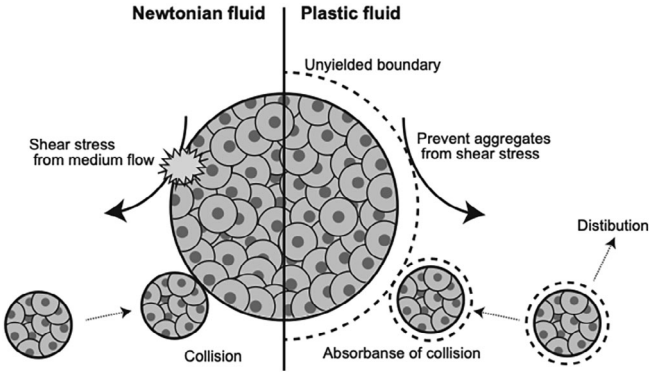

FIGURE 7 Schematic drawing of the mechanism of improving growth yield in iPSC suspension culture, which is suggested by the results

TkDN-4M, improvement of cell growth in high agitation condition occurred same as 15M63 but the prevention of the fusion of aggregates in slow shaking condition did not occur (Figure S5).

4 | DISCUSSION

The culture of hiPSCs in suspension has previously been used to obtain sufficient cells for various applications such as regenerative therapy and drug screening. During suspension culture, cells form aggregates and tend to settle; thus, agitation is required to avoid sedimentation and to produce a stable suspension. However, their low robustness to shear stress limits maximum agitation.[23,24] For these reasons, optimization of the agitation rate is essential, and some hiPSC lines have no optimum rate of agitation, owing to their low robustness to shear stress. Various attempts have been made to accomplish ideal agitation, with size-controlled aggregation, for example, by the development of novel culture vessels.[11] In this study, we modified the viscometric properties of the suspension culture medium to improve the robustness of the hiPSC aggregates to agitation.

According to growth analyses, hiPSCs in our Bingham plastic fluid realized a high fold-increase in an extensive range of agitation speeds, from slow (60 rpm) to fast (120 rpm). Generally, agitation causes shear stress, leading to cell damage and death, which is supported by the dead cell count, based on LDH released from cells (Figure 6). In conventional culture media, this low robustness to shear stress limits the agitation rate, which is then insufficient to keep aggregates floating, in the case of some iPSC cell lines. According to the results of fast agitation, hiPSCs attained higher tolerance to high shear stress in the Bingham plastic medium. Although the exact reason for this is unclear, it is expected that an unyielded boundary layer appears on the surface of aggregates, affording some protection from shear stress (Figure 7). Although there are few studies on culturing cells in Bingham plastic media, some numerical studies have suggested that an unyielded region appears to encapsulate objects placed in the flow.[25-27] We attempted to observe the unyielded layer surrounding aggregates by micro PIV and fluorescence-based localization. These attempts did not clarify the existence of an unyielded layer (data not shown), but any such layer would have been quite thin (<10 μm).

This research suggested that the different approach to reduce hydrodynamic damage during suspension culture. In a few decades, there were many researches about viscosity modification to reduce fluid mechanical damage on cells.[16-19,24] Many researchers added the various polymers to the medium for improving cell viability. These polymers increased the viscosity (from 3 to 30 mPa·s) to prevent medium flow causing cell damage.[16] However, high viscosity medium has a difficulty of medium handling such as separation and mixture. On the other hand, Bingham plastic is easier to handle due to lower viscosity in high shear rate and expected to protect cells from shear stress due to high viscosity in low shear rate.

The Bingham plastic medium also improved cell growth at a low agitation rate (60 rpm) due to the prevention of excess aggregation and collisions, which is supported by morphological observations (Figure 4). According to the distribution experiment with stained aggregates, non-Newtonian fluid maintained distribution better than the Newtonian medium. In Newtonian fluid, with orbital shaking, surface flow from the center to periphery occurred, due to centrifugal force. Then, at the bottom, flow occurred from the periphery to the center, so that cells settled down to the bottom and gathered in the center. This phenomenon was reported a hundred years ago, and is known as Einstein's tea-leaf paradox.[22] This phenomenon promotes cells to aggregate and can cause large aggregates to form.[11,12] In contrast, the observation of aggregate distribution in non-Newtonian fluid showed that they were well distributed from center to periphery and from bottom to surface. This was because the Bingham plastic fluid, with yield stress, prevented sedimentation, and aggregates were transported with the flow at both surface and bottom. Due to the

more even distribution of aggregates, there were fewer collisions in suspension culture. Thus, the decrease in collisions limited coalescence and increased growth at lower shaking speeds (60 rpm).

In a comparison between cultures with and without pre-aggregation, the pre-aggregation step improved the risks of agitation. However, the improvement was not perfect, and growth decreased at sub-optimal rates of agitation. Culturing cells in a Bingham plastic fluid, after initial aggregation, improved cell growth, and similar growth and death data were obtained across the complete range of 60–120 rpm (black bars in Figures 5 and 6). However, from the preliminary experiment, a single hiPSC suspension in the Bingham plastic medium formed one-third fewer aggregates than in the conventional culture medium (Figure S2). It is speculated that the unyielded boundary layer reduces collision and thus inhibits initial aggregate formation. Therefore, the Bingham plastic medium supplementation was more effective after an initial aggregation of 1 to 2 days.

Bingham plastic fluid medium has a higher viscosity and yield stress than normal media, which might prevent the mixing of the culture medium and limit the transportation of substrate and oxygen. Generally, pluripotent stem cells generate their energy via glycolysis, which does not require oxygen, so their oxygen requirement is low. According to histological analysis with HE staining, the Bingham plastic fluid did not affect the oxygen supply to the aggregates in a 4 ml volume of suspension culture (Figure S4). However, oxygen depletion (lower than 1%) delays proliferation[28]; thus, a further aeration strategy is probably required when scaling up the culture system (>1 L), for example, a culture vessel with a gas-permeable membrane improves oxygen supply.[11]

These effects of the Bingham plastic medium are partly dependent on the cell line. We performed the same experiments for cell growth and death on two cell lines, 15M63 and TkDN-4N. With TkDN-4N the prevention of aggregation at a slow agitation rate did not occur, and large aggregates were formed (Figure S5a). This difference was probably due to the individual characteristics of each hiPSC line, such as adhesion ability. Cell aggregates in suspension culture show behaviors such as fusion and collapse, and these behavioral tendencies vary between cell lines.[29] However, both iPSC lines showed higher tolerance to shear stress in the Bingham plastic medium (Figures S5b and S5c), which suggests that the relaxation of shear stress is independent of cell characteristics.

In this study, we showed that a Bingham plastic medium improves resistance to shear stress in suspension culture. As mentioned, agitation in suspension culture is a tradeoff between preventing sedimentation and cell damage from shear stress. In particular, hiPSCs are sensitive to shear stress; thus, agitation is limited by shear stress. Therefore, culturing in a Bingham plastic fluid is helpful to developing a stable system for mass culture.

5 | CONCLUSION

We demonstrated the potential of Bingham plastic media to improve the propagation of a suspension culture of hiPSCs, which are sensitive to shear stress and require controlled aggregation. The results at 120 rpm showed that Bingham plastic fluid protected cells from strong shear stress during agitation. In addition, Bingham plastic fluid improved aggregation in suspension culture. These modifications positively affected the cell yield and showed that Bingham plastic medium is a promising development for the suspension culture of hiPSCs.

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PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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