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Utility of a rotation/revolution-type agitator for chondrocyte isolation during preparation of engineered cartilage

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During the manufacture of cell- and tissue-based products, such as engineered cartilage for autologous chondrocyte implantation, maximizing the number of cells isolated from donor tissue substantially improves the productivity of these products. The method used for agitating tissues with digestive fluid and enzymes can considerably affect both the quality and quantity of isolated cells. This study aimed to investigate the effectiveness of a rotation/revolution-type agitator for chondrocyte isolation following the enzymatic digestion of rat costal cartilage. Cartilage tissue cut into 1 mm^3-thick sections was equally divided between two groups and placed in 50-mL conical tubes; sections in both groups were digested using 0.1 mg/mL liberase TH (collagenase/thermolysin) at 37 °C for 4 h with either rotation/revolution or conventional orbital agitation method. Compared with using conventional orbital agitator, using the rotation/revolution-type agitator resulted in a significant (>two-fold) increase in the number of isolated cells. In subsequent primary cultures, chondrocytes obtained by rotation/revolution agitation showed superior initial attachment to tissue culture dish on day 1 and 2 compared with those obtained by conventional agitation; however, no differences in cell proliferation or cartilage-related molecule expression patterns were observed between cells derived from either method after 3 days of subculture. These findings suggested that there are no disadvantages to the proposed rotation/revolution agitation method. Rotation/revolution-type agitators are a promising apparatus for preparing chondrocytes for primary cultures and cartilage tissue engineering.

[**Key words:** Rotation/revolution; Cell isolation; Chondrocyte; Optimization; Culture]

Normal articular cartilage, known as hyaline cartilage, has abundant extracellular matrix (ECM), primarily composed of type II collagen, hyaluronan, aggrecan, and several glycoproteins surrounding individual chondrocytes encapsulated by cartilage lacunae. These components of the ECM contribute to the specific physiological properties and biomechanical functions of the articular cartilage (1). However, the articular cartilage is avascular, which may be attributed to the its limited intrinsic healing capacity in response to an injury. Common procedures used for cartilage restoration, such as drilling, microfracture surgery, and abrasion arthroplasty, occasionally succeed in reconstructing the articular cartilage by recruiting bone marrow-derived mesenchymal cells; however, this is temporary and eventually results in the formation of fibrous cartilage (2–5). Osteochondral autografting (mosaicplasty) is another technique for cartilage repair; however, fibrous cartilage formation is induced at the surface of the donor holes in the grafted tissue (6).

The first clinical trial of autologous chondrocyte implantation (ACI) was reported in 1994 (7). ACI was a groundbreaking treatment that involved the use of cell suspension injections; however, it had several limitations owing to the use of monolayer-cultured chondrocytes. Subsequently, over the years, many clinicians and researchers contributed to improvements in ACI. Matrix-assisted ACI, using engineered cartilage embedded in collagen gel, is expected to be a promising approach for the repair of injured knee cartilage (8–11). This treatment can be applied to traumatic cartilage defects of approximately 4–6 cm^2 in size. Typically, preparing tissue-engineered cartilage involves several processes including cell isolation (primary monolayer culture), three-dimensional (3D) culture (using collagen gel to avoid changes in cell phenotype (12)), and quality analysis at each stage. Generally, the end product is prepared using cells obtained from a limited volume of cartilage (approximately 0.3–0.5 g). Thus, the application of an enzymatic digestion process that isolates the maximum number of normal chondrocytes from the excised tissue is a key step in this technology. The reaction mixture, including cartilage tissue fragments and enzymes, is stirred or shaken to efficiently collect the cells as agitation using a high enzyme concentration reduces the digestion time. Moderate agitation may cause lower cell yields, whereas vigorous agitation can result in intense cell damage and low cell viability due to physical stress. Therefore, applying agitation at this stage can influence cell viability, phenotype, and yield in cartilage tissue engineering.

The rotation/revolution-type agitator (Fig. 1A) is a useful apparatus to uniformly mix even high viscosity fluids and powders

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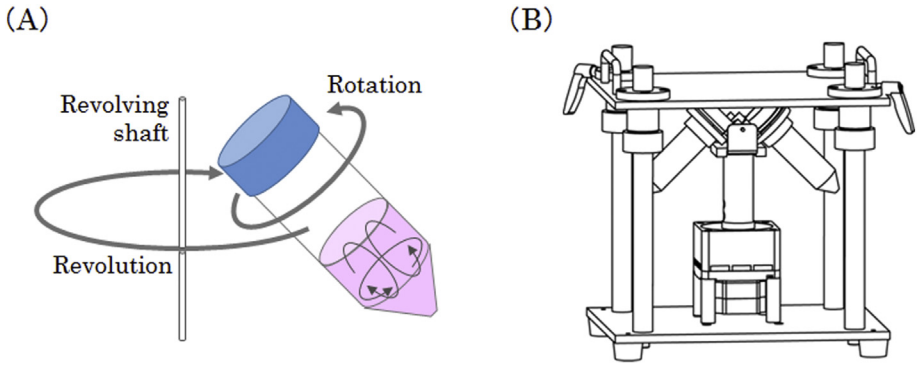

FIG. 1. Schematic diagrams of the rotation/revolution-type agitator. (A) Driving mechanism of agitation within the tube. (B) External view of the prototype used in this study.

without the physical destruction of particles through the use of stirring elements such as paddles (13–15). Methods involving such agitators are also suitable for emulsifying and agitating samples without incorporating air bubbles (14,16,17) and are utilized in various research and industrial procedures, including semiconductor manufacturing. However, such methods are seldom used in the biomedical and life sciences, aside from in prosthodontics. In this study, the effects of different agitation methods on cell isolation during cartilage tissue culture were assessed. We demonstrated that the rotation/revolution agitation method is more effective than the conventional orbital agitation method for the isolation of chondrocytes from cartilage tissue, especially regarding the number of viable cells obtained from equal tissue volumes.

MATERIALS AND METHODS

Equipment design A rotation/revolution-type agitator within in an incubator controlled at 37 °C was specially designed to accommodate commercially available 50-mL conical tubes (Fig. 1B). The angle between tube and the drive shaft was 56.1°, and the ratio of rotation to revolution was adjusted to 1.4. The revolution speed was set to 120 rpm; in this condition, surface of the liquid in the tube was approximately orthogonal to the rotation axis of the tube during agitation. MMS-3020 multi-functional shaker (EYELA, Tokyo, Japan) placed within the incubator controlled at 37 °C was used in the orbital mode at 120 rpm for comparison. The state in which tissue moved within each tube during agitation was recorded as a digital image.

Physical cell damage assay (simple hemolysis assay) As recommended in the International Organization for Standardization (ISO10993-4) (18), hemolysis assay is an effective screening test to evaluate blood cell damage caused by material characteristics and the physical and chemical effects of blood-contacting devices. These effects are assessed by the measurement of erythrocyte membrane fragility. Therefore, to evaluate the effect of agitation methods on physical cell damage during isolation, simple hemolysis assays using erythrocytes were performed as previously described (19). A test solution containing erythrocytes was prepared from commercially available defibrinated rabbit blood (Kohjin-Bio, Sakado, Japan), and cartilage pieces (approximately 1-mm^3 thick) were added to the solution (0.4 g per 10 mL of the solution). The degree of hemolysis following agitation at 37 °C for 4 h in each tube was assessing by measuring the absorbance at 576 nm (A_{576}) according to the following formula:

$$\text{Hemolysis ratio (\%)} = [(A_{576} \text{ of sample}) - (A_{576} \text{ of negative control})]/[(A_{576} \text{ of positive control}) - (A_{576} \text{ of negative control})] \times 100 \quad (1)$$

Static-conditioned samples and sonicated samples were used as negative and positive controls, respectively.

Isolation of normal chondrocytes All animal experiments in the present study were performed with the prior approval of the Animal Care and Experiment Committee at Tokyo Denki University. In this study, rat costal cartilage samples were used to compare enzymatic digestion efficiency between the two agitation methods. Hyaline cartilage was collected from the ribs of Sprague–Dawley rats. Samples was immersed in 0.1% trypsin at 37 °C for 30 min to remove fibrous tissue. After rinsing three times with Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS), cartilage samples were cut into 1 mm^3–thick sections. These sections were equally divided between two 50-mL conical tubes (approximately 0.3–0.4 g of tissue per tube) and digested at 37 °C for 4 h in 10 mL of Dulbecco's modified eagle medium (DMEM)/F12 containing 0.1 mg/mL liberase TH (containing collagenase and thermolysin, Roche) using either the rotation/revolution agitation method or the conventional orbital agitation method described earlier. Cells isolated using both the agitation methods were individually passed through a 70-μm mesh cell strainer; undigested tissues were weighed using an AUX-320 electronic balance (Shimadzu). Tissue digestibility was determined based on the proportion of reduced mass to original wet weight of the divided cartilage.

Isolated cells were collected via centrifugation, and suspended in fresh DMEM/F12 medium containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. Total viable cells were counted following trypan blue staining.

Primary chondrocyte culture Isolated normal chondrocytes were suspended in DMEM/F12 containing 10% FBS and ITS (10 μg/mL insulin, 6.7 ng/mL transferrin, 5.5 μg/mL selenium). Cells were seeded at a density of 2.0×10^4 cells/cm^2 in a 12-well culture plate and incubated for 72 h at 37 °C with 5% CO_2 to evaluate cell proliferation and gene expression.

DNA quantification At each time point during primary chondrocyte culture, cells were rinsed with PBS and homogenized in 10 mM Tri-HCl (pH 7.5) containing 1 mM EDTA and 0.01% sodium dodecyl sulfate for DNA quantification. Double-stranded DNA was stained using the Quant-iT PicoGreen dye (Invitrogen), and stained DNA were assessed using an F-4500 fluorescence spectrophotometer (Hitachi High-Technologies, Tokyo, Japan) according to manufacturer's instructions.

Reverse transcription-polymerase chain reaction Reverse transcription-polymerase chain reaction (RT-PCR) was performed as previously described (20). Briefly, total RNA was extracted from cells using an ISOGEN II RNA isolation kit (Nippon Gene, Tokyo, Japan) using deoxyribonuclease I treatment. RNA samples were reverse transcribed into cDNA using a SuperScript II first-strand synthesis system for RT-PCR (Invitrogen). To analyze the expression of specific genes, equal volumes of first-strand cDNA were amplified via PCR using a TaKaRa Ex Taq kit (Takara Bio Inc., Ohtsu, Japan) with specific forward and reverse primers for each gene. The primers used were as follows: SRY Box 9 (Sox9; Genbank accession number NM_080403), forward: 5′-TCCTAACGCCATCTTCAAGG-3′, reverse: 5′-CGGCAGGTATTGGTCAAACT-3′; aggrecan (Acan; Genbank accession number NM_022190), forward: 5′-AAGGACGAGTTCCCTGGAGT-3′, reverse: 5′-TGTAGCAGATGGCGTCGTAG-3′; collagen type I alpha 1 chain (Col1a1; Genbank accession number NM_053304), forward: 5′-CTGCAACAAATCCCCACACA-3′, reverse: 5′-CCACCCCTTCACAGAGATGT-3′; collagen type II a 1 chain (Col2a1; Genbank accession number NM_012929), forward: 5′-TTCTGGTAACCCAGGGACTG-3′, reverse: 5′-ACCTGCCAGACCATCTTGAC-3′; beta-2 microglobulin (B2m; Genbank accession number NM_012512), forward: 5′-CCGTGATCTTTCTGGTGCTT-3′, reverse: 5′-TTTTGGGCTCCTTCAGAGTG-3′. PCR conditions were as follows: denaturation for 1 min at 94 °C, annealing for 30 s at 55 °C, elongation for 30 s at 72 °C; this was repeated for 30–32 cycles. DNA amplified using PCR was subjected to electrophoresis in 2% agarose gel; digital photographs of ethidium bromide-stained gels were obtained using an ImageQuant LAS4000 image analyzer (GE Healthcare).

Western blot analysis Cells were lysed in 20 mM Tris–HCl (pH7.5) containing 1% Triton X100, 5 mM EDTA-2Na, 1 mM 4-amidinophenylmethanesulfonyl fluoride, 2 μg/mL of leupeptin, 2 μg/mL pepstatin. Protein concentration in each sample was measured using the bicinchoninic acid method. Equal volumes of total protein extracts were subjected to sodium dodecyl sulfate-acrylamide gel electrophoresis and electro-blotted onto a polyvinylidene difluoride (PVDF) membrane. The membrane was then blocked with 1% bovine serum albumin (in PBS) for 30 min at room temperature, then incubated overnight at 4 °C with primary antibodies against rat SOX9 (ERP14335, Abcam), Aggrecan (ABT1373, EMD Millipore), type II collagen (bs-0709R, Bioss), and GAPDH (EPR16884, Abcam). To visualize the proteins of interest, alkaline phosphatase-conjugated secondary antibody and corresponding substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) were used.

Statistical analysis The Student's unpaired *t*-test or the Aspin–Welch paired *t*-test were employed for equal variance or unequal variance analyses, respectively. Significant differences were determined at $p < 0.05$ for the *t*-test.

RESULTS

Agitation of cartilage tissue Prior to enzymatic digestion of the cartilage tissue, tissue movement in the tube during rotation/revolution agitation was observed and estimated for comparison with that in the tube during conventional orbital agitation. During convention orbital agitation, most tissue remained at the bottom of the tube, whereas the liquid surface was considerably disrupted (Fig. 2A–F). This was consistently observed irrespective of the angle (vertical or diagonal setting) of the tube. In contrast, in the rotation/revolution agitation method, although the tissue accumulated at the bottom side wall of the tube in static conditions (Fig. 2G), it was began floating immediately after the agitation began (Fig. 2H, I).

Detection of physical cell damage during tissue agitation To evaluate physical damage to cells during agitation, simple hemolysis assays were conducted in the presence of cartilage samples to provide an environment conductive for damage during the cell isolation process (Fig. 3). No hemolysis was detected after rotation/revolution agitation, whereas moderate hemolysis was observed after orbital agitation. A significant difference was noted between the two methods.

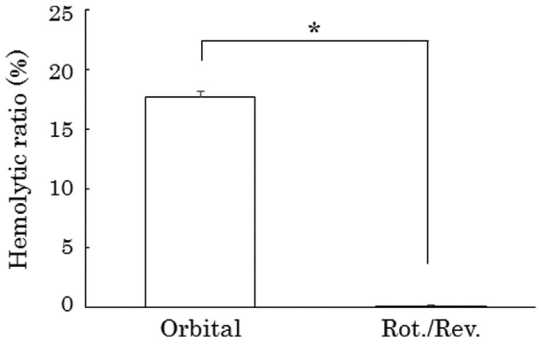

FIG. 3. Comparison of physical damage to cells during agitation using hemolysis assay. No hemolysis was observed in the rotation/revolution agitation group (Rot./Rev.), whereas moderate hemolysis was detected in the conventional orbital agitation group. Data are shown as mean ± standard error (SE) (n = 3), $^*p < 0.001$.

Isolation of chondrocytes using a rotation/revolution-type agitator Enzymatic digestion efficiency during chondrocyte isolation from cartilage tissue using rotation/revolution agitation was compared with that using conventional orbital agitation

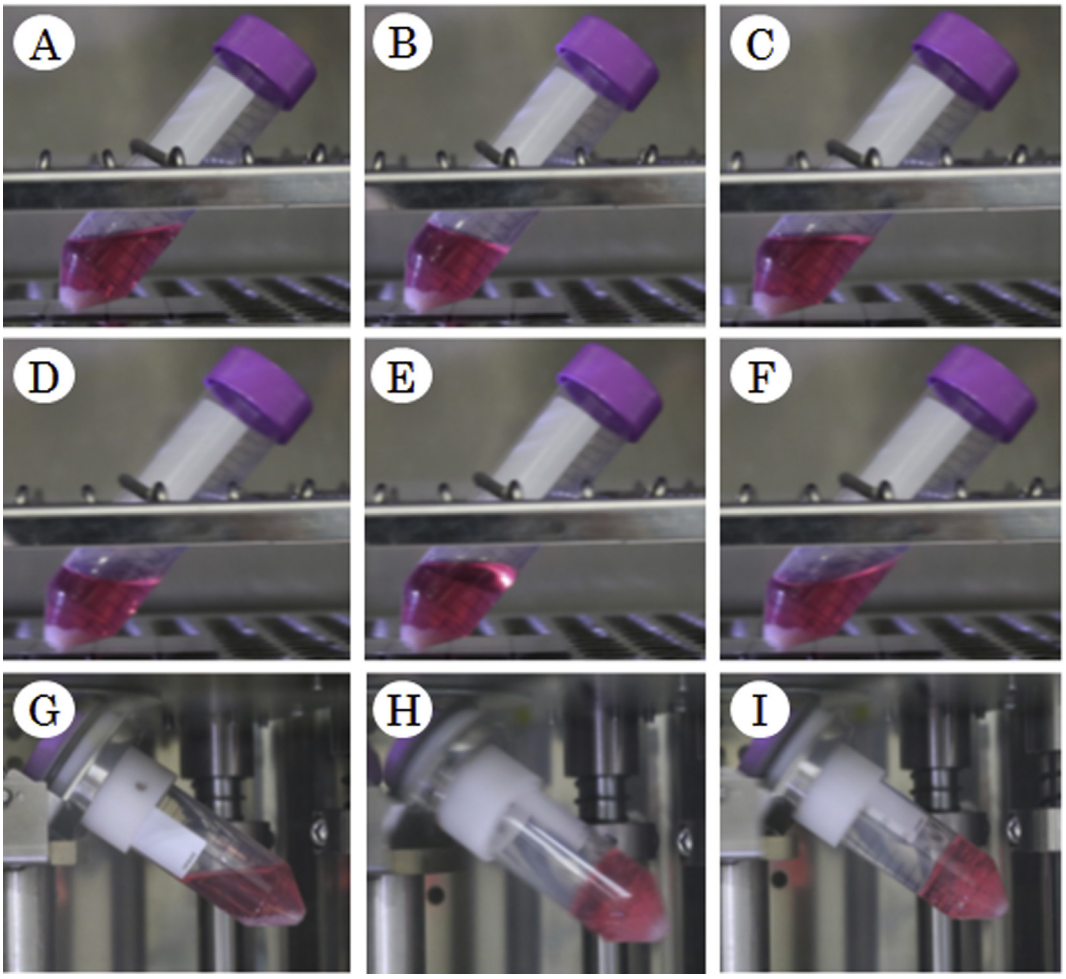

FIG. 2. Photographs showing the distribution of cartilage pieces within the tube during orbital agitation (A–F) and rotation/revolution agitation (G–I). Approximately 0.4 g of cartilage pieces was added to 10 mL of the test solution. Serial photographs were obtained every 1/6 s during conventional orbital agitation at 120 rpm (A–F), and tissue was seen to accumulate at the bottom of the tube. Aside from the static state (G), the distribution of floating tissue in the center of the tube was confirmed during rotation/revolution agitation at 120 rpm (H, I).

during a 4 h period. It was found that 76.3% of cartilage was enzymatically digested in the rotation/revolution agitation group, whereas only 65.1% of cartilage was enzymatically digested in the conventional orbital agitation group (Fig. 4A). Furthermore, the number of isolated cells was approximately 1.2×10^7 and 4.3×10^6 cells/g of cartilage tissue in the rotation/revolution and conventional orbital agitation groups, respectively (Fig. 4B). Thus, the rotation/revolution agitation method yield approximately 2.5-fold more cells compared with the conventional orbital agitation method from equal initial tissue weights.

Proliferation of isolated chondrocytes Because effects of this novel agitation method on cell proliferation and gene expression in isolated primary chondrocytes was previously unknown, the proliferation pattern of chondrocytes isolated using the two agitation methods was evaluated using DNA quantification as an index, as shown in Fig. 5A. On day 1 and 2, the DNA content of attached cells isolated using the rotation/revolution-type agitator was significantly higher than that of those isolated using the conventional orbital agitator. On day 3, DNA content increased and was relatively high in the rotation/revolution agitation group but was not significantly different from that in the conventional orbital agitation group. In addition, no morphological differences were noted in primary cultured cells between the groups on any of the 3 days (Fig. 5B).

Cartilage-related gene expression in primary chondrocytes Cartilage-related gene expression in primary chondrocytes was assessed using RT-PCR. As shown in Fig. 5C, Sox9, aggrecan, and type II collagen were detected in both groups as representative positive markers of hyaline cartilage chondrocytes; however, no type I collagen, a marker of dedifferentiation to fibrous cartilage chondrocytes, was noted. Positive marker proteins detected using RT-PCR were confirmed by Western blotting using specific antibodies as described in the methods section (Fig. 5D).

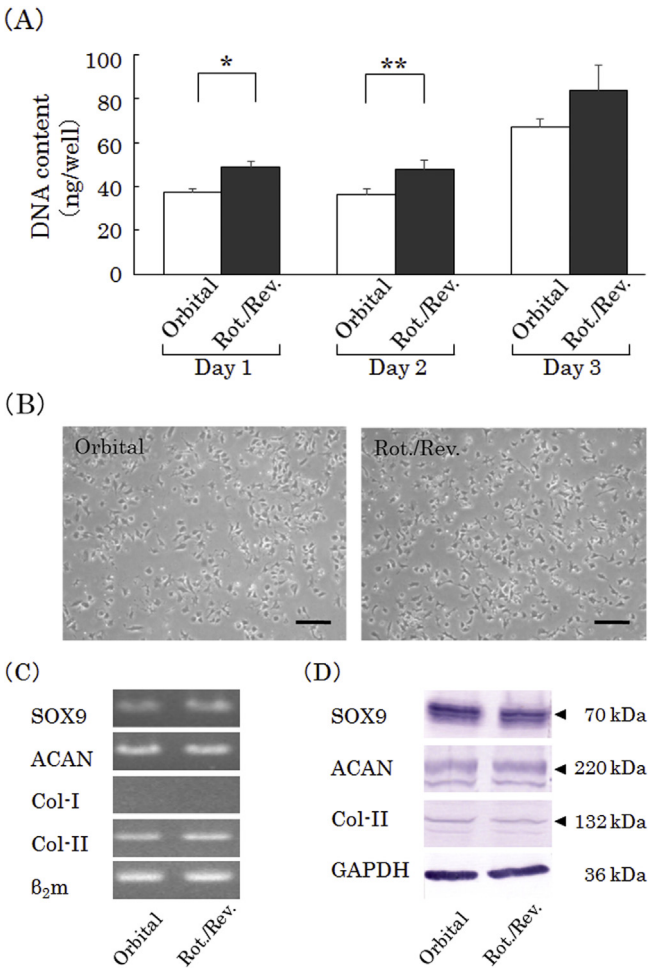

FIG. 5. Effects of agitation method on (A) cell proliferation, (B) cell morphology, and expression of (C) mRNAs and (D) proteins of the isolated chondrocytes. (A) Cell proliferation pattern in each group was evaluated by assessing the DNA content. Data are shown as mean ± SE (n = 3) (*$p < 0.01$, **$p < 0.05$). (B) Phase-contrast microphotographs of chondrocytes were taken on day three of primary culture. Scale bar: 200 μm. (C, D) Comparison of expression patterns of cartilage-related molecules in primary chondrocytes on day 3 were analyzed. Typical expression patterns observed using (C) RT-PCR and (D) Western blot analysis.

DISCUSSION

Several studies on 3D culture have made great contributions to cartilage tissue engineering (12,21–25). However, there have been very few studies concerning the optimization of cell isolation processes (26,27), and there has been scarce investigation regarding the importance of agitation of the cartilage and enzyme mixture during the cell isolation process. Maximizing the number of viable chondrocytes obtained from a limited volume (generally < 0.4 g) of available cartilage is considerable challenge, which must be overcome to improve the manufacturing of engineered cartilage. The optimization of chondrocyte isolation can substantially contribute to obtaining sufficient cells to prepare a reliable product. In the present study, we focused on comparing the effects of two different agitation methods (novel rotation/revolution agitation and conventional orbital agitation) on cell isolation efficiency and demonstrated that the rotation/revolution agitation method was

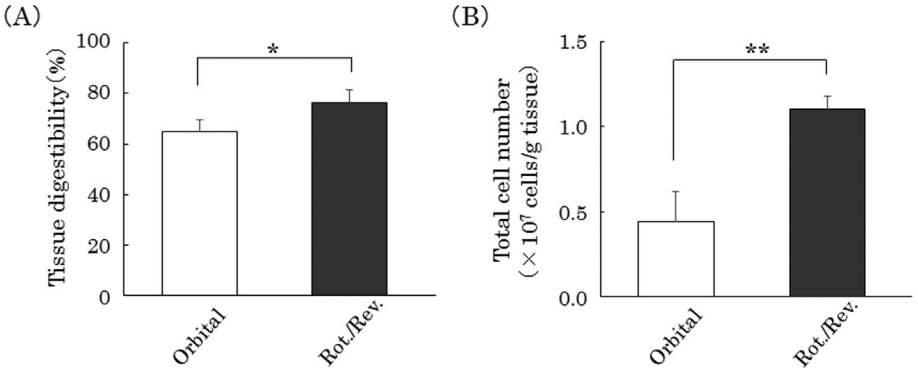

FIG. 4. Effects of agitation method on (A) tissue digestibility and (B) cell yield. Tissue digestibility and cell yield in the rotation/revolution agitation group (Rot./Rev.) were significantly higher than in the orbital agitation group (at the level of *$p < 0.01$ and **$p < 0.05$, respectively). Data are shown as mean ± SE (n = 3).

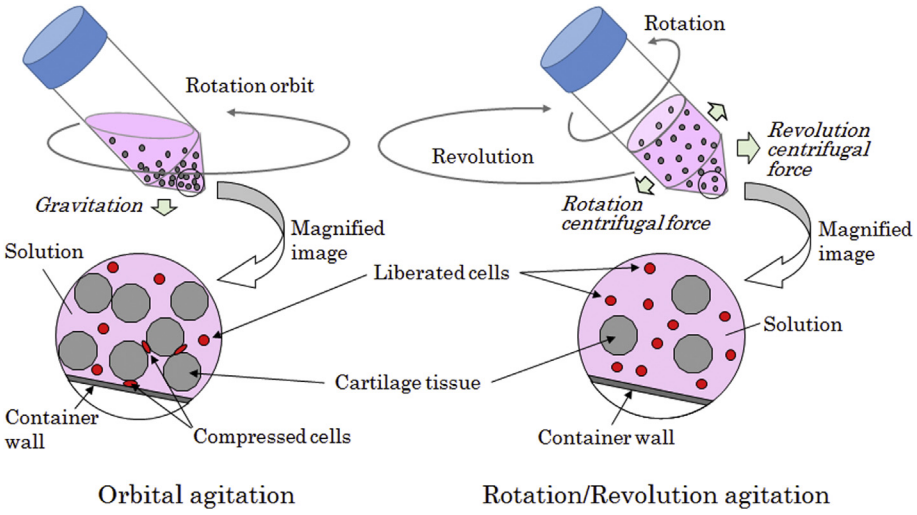

FIG. 6. Schematic diagrams of the typical distribution of cells and tissues in tubes during agitation. There may be many compressed and damaged cells in the tube of the orbital agitation group, but not in the rotation/revolution agitation group.

superior to the conventional orbital agitation method regarding mixing efficiency, tissue digestion ratio, and the number of viable chondrocytes isolated. Although several functions can be applied to an ideal experimental model, investigating the fluid dynamics of cells and enzyme solution in 50 mL conical tubes during rotation/revolution agitation is difficult. This is because of conditions such as fluid volume, fluid viscosity (concentration), particle density (cell and tissue), and rotation speed result in different complex flow of tissues. In addition, there is little evidence of these patterns even during orbital shaking. Therefore, our visualized images present evidence supporting different flow patterns of tissues during the agitation types (Fig. 2).

It is worth noting that approximately 1.2×10^7 viable cells/g of cartilage tissue (Fig. 4B) were isolated using rotation/revolution agitation in a relatively short treatment time (suggesting the maximum achievable cell number) compared with the typical number (approximately 10^6 cells/g of cartilage tissue) obtained using conventional orbital agitation in the treatment same time (26,27). This increase in both digestion rate and cell yield in the rotation/revolution agitation group is attributed to differences in physical damage to cells, as shown in Fig. 3. When both tissue fragments and free cells were present, cells and tissue pieces gathered at the bottom of the tube during conventional orbital agitation, under the influence of gravity (Fig. 2A–F); it was suggested that physical damage to cells due to collision, friction, and compression from the tissue fragments was reflected in the hemolysis assay (Figs. 3 and 6). Conversely, cells and tissue pieces floated in solution during rotation/revolution agitation as shown in Fig. 2H and I. This agitation method did not induce cell damage, as no hemolysis was observed even when both erythrocytes and cartilage fragments were included in the tube during agitation (Fig. 3). In our hemolysis assay, we used oxygenated hemoglobin as an index of hemolysis (absorbance at 576 and 540 nm), and similar results were obtained when measuring hemolysis of reduced hemoglobin (absorbance at 415 nm).

In tissue and primary cultures, apart from the number of cells obtained, the characteristics of isolated cells are also crucial. Therefore, it is necessary to evaluate the effects of the agitation method and physical damage during cell isolation on initial cell proliferation and gene expression. The total DNA content in adhered cells was correlated with the number of cells adhered, and the rotation/revolution agitation group showed a significantly higher DNA content than the conventional orbital agitation group on days 1 and 2. However no significant difference was noted between the groups on day 3 of primary culture (Fig. 5A). These data suggest that the differences in physical damage during agitation affect initial cell adhesion but do not affect subsequent proliferation once adhesion had been established. In addition, because gene and protein expression patterns of primary cultured cells were nearly identical between both the groups (Fig. 5C, D), the rotation/revolution-type agitator was confirmed to be a useful apparatus for chondrocyte isolation in cartilage tissue engineering.

In conclusion, cell isolation from tissue is the first key step in manufacture tissue-engineered products. The present study demonstrated that different agitation methods lead to differences in the number of viable cells recovered and that rotation/revolution agitation method is a more useful method for cell isolation than conventional orbital agitation method. Furthermore, the rotation/revolution-type agitator used in this study can be used in studies involving human articular cartilage as the amount of cartilage used in this study was approximately of the same scale as that used in clinical protocols.

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