Journal of Bioscience and Bioengineering
VOL. 133 No. 1, 83–88, 2022

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# Internal radial perfusion bioreactor promotes decellularization and recellularization of rat uterine tissue

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Received 14 July 2021; accepted 12 September 2021
Available online 19 October 2021

**The advances in infertility treatment technologies such as in vitro fertilization (IVF) help many infertile women to be able to get pregnant. However, these infertility treatments cannot be applied to women who are suffering from absolute uterine factor. Fabrication of functional scaffold in tissue engineering approach is believed to play an important role for uterine regeneration and uterus replacement for treating absolute uterine factor infertility. In this research, we developed an internal radial perfusion bioreactor to promote decellularization and recellularization for fabrication of functional engineered uterine tissue. As a result, the DNA contents of the decellularized uterine tissue with high hydrostatic pressure followed by 7 days internal perfusion washing decreased by 90% compared to native tissue. Collagen and proteoglycan contents in the pressurized uterine tissue with the internal perfusion bioreactor, static (control) and shaking treatment with high hydrostatic pressure showed no significant change compared to the native tissue. The newly developed perfusion bioreactor also enabled to recellularize in the decellularized tissue with statistically significant increase of DNA by 614% compared to non-seeded cell groups. Vimentin and 4',6-diamidino-2-phenylindole (DAPI) was homogeneously expressed in the seeded endometrial stromal cells in the recellularized tissue fabricated using the bioreactor. With the developed internal radial perfusion bioreactor, we are the first group to successfully recellularized uterine tissue in all layers including epithelium, endometrium and myometrium. These results showed that the internal perfusion bioreactor has potential to be utilized for fabrication of functional engineered tissue to promote tissue regeneration.**



[**Key words:** Recellularization; Decellularization; Perfusion bioreactor; Tissue engineering; Scaffold; Uterus]

Infertility refers to the failure to achieve a clinical pregnancy which caused from various reasons such as damaging of fallopian tubes, ovulatory problems, endometriosis and conditions affecting the uterus. More than 7.5 million women in the world at reproductive age are suffering from the infertility. About 50% of couples obtaining assisted reproduction technology still cannot get pregnant especially for patient with absolute uterine factor infertility, with a prevalence around 10% in women age between 33 and 40 years old (1–3). Recent successful of organ transplantation in human showed the promising results to overcome difficulties in achieving natural pregnancy. Uterine transplantation restores reproductive anatomy in women as well as allows the opportunity to conceive, experience gestation and acquire motherhood (2,4,5). However, the transplanted organs have the risk of allograft rejection. Immunosuppressive drugs are considered as an obligation to prevent graft rejection after allogeneic organ transplantation, nevertheless long-term use of immunosuppressive regimens have cumulative side effects such as cardiovascular disease, metabolic disease, cancers and infections (6,7). For those who are suffering from the infertility due to the uterus malfunction, a functional scaffold in the tissue engineering approaches is believed to play an important role for the uterus transplantation (8).

Tissue engineering has emerged as a promising approach to develop identical and functional tissues to implant into its specific organ. Briefly, the engineered tissue composes of viable cells seeded on native structure-like scaffold. However, achieving functional engineered tissue prior to organ transplantation remains a significant challenge for tissue engineering. Decellularization is a technique to fabricate three-dimensional extracellular matrix as a scaffold by removal of the cellular components as well as antigenic epitopes of the organ while preserving the natural native-like macro- and microstructure (7). Decellularization has been widely used for scaffolding in tissue engineering such as heart, cartilage or nerve allograft (9,10). Although, sodium dodecyl sulfate (SDS) is widely used as a conventional method for decellularization due to efficiency of cell removal, this method critically deconstructs and denatures extracellular matrix components. Recently, mechanical approaches such as high hydrostatic pressure have been applied for fabrication of decellularized extracellular matrix. Previous research showed that high hydrostatic pressure could improve maintaining of decellularized extracellular matrix structure compared to conventional chemical approach (11–13). However, to remove cell

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1389-1723/$ – see front matter 
https://doi.org/10.1016/j.jbiosc.2021.09.007

residuals for decellularized extracellular matrix fabricated by high hydrostatic pressure, washing process is required (14).

For tissue and organ transplantation, recellularization is required for engineered functional decellularized scaffold. Recellularization is a process of seeding cells in order to redistribution of cells similar to their spatial configuration as well as improve in vivo organ regeneration and function. However, repopulation with an appropriate number of cells, and placement of these cells to necessary niches within the scaffold to match the native distribution as much as possible is remained challenges (7). Current methods for recellularization of decellularized uterine tissue include direct cell culture on the scaffold under static condition and perfusion culture. Recent research showed that seeded cells were remained on the surface of uterine tissue with low penetration rate in the tissue and spatial redistribution of seeded cells (15–17). Hence, the recellularization of thick and anatomically complexity structure tissue with sufficient cells in all layers of the tissue is required for an ideal tissue-engineered model. In order to achieve this, we design and develop an internal radial perfusion bioreactor to promote the decellularization and recellularization process in all layers of uterine tissue.

## MATERIALS AND METHODS

**Preparation of uterine tissue** All animals used in this experiment were conducted under approval by the University of Tokyo animal experiment committee. The title of the approved animal experiment plan is "Rat uterine tissue engineering" (ID number is KA17-9-1). Preparation of uterine tissue was aseptically performed with 9 weeks old female Sprague Dawley rats. Rats were sacrificed by overdose of chemical anesthetics such as isoflurane inhalation solution. Twenty-five millimeters of uterine horns were extracted, and then trimmed connective tissue and fat off. The uterine samples were rinsed with phosphate buffer saline (PBS).

**High hydrostatic pressurization of uterine tissue** The uterine tissue was double packed with polyethylene bag in PBS solution. In this study, high hydrostatic pressure was applied by using a cold isostatic pressurization machine (Dr. CHEF; Kobelco, Kobe, Japan). The chamber was pressurized to 980 MPa with 65.3 MPa/min at 30 °C, and then held for 10 min before being reduced back to atmospheric pressure at 65.3 MPa/min (11,12).

**Washing process for removal of DNA** The washing buffer contained 0.9% NaCl (Wako, Tokyo, Japan), 0.05 M magnesium chloride hexahydrate (Wako), 0.2 mg/ml DNase I (Roche, New York, NY, USA) and 1% penicillin and streptomycin (Gibco, Tokyo, Japan). The washing process was carried out in three different conditions: static, shaking, and bioreactor. For static treatment, samples were washed for 1 week at 4 °C in a washing buffer. For shaking treatment, samples were placed on commercially available shaker with 1 Hz for 1 week at 4 °C in a washing buffer (Fig. 1D).

**Perfusion system design for decellularization** To decellularize tissue, perfusion with DNase I was introduced after mechanical pressurization with high hydrostatic pressure. The schematic diagram of internal radial perfusion bioreactor for decellularization is shown in Fig. 1E. The washing process with DNase I was prepared by setting 25 mm length of pressurized uterine tissue on tissue frame covered with 0.22 μm baffle on the top of polypropylene frame stand. Then, the tissue frame was set on bioreactor filled with 0.2 mg/ml DNase I solution. To prevent mixing of solution during perfusion, DNase I solution and milliQ water were blocked by silicone membrane between tissue chamber and water chamber. Infuse/withdraw-continuous syringe pump (Legato, KD Scientific, Holliston, MA, USA) was performed to pressurize and generate the internal perfusion through water chamber by optimized setting the syringe pump to 0.1 Hz with 1500 μl/min infuse/withdraw rate. The bioreactor was processed in sterile incubator at 4 °C for 7 days.

**Static culture for recellularization** Static culture (control) was used as a conventional method for cell seeding on decellularized extracellular matrix. Briefly, approximately $1.0 \times 10^7$ cells of rat endometrial stromal cells were directly seeded

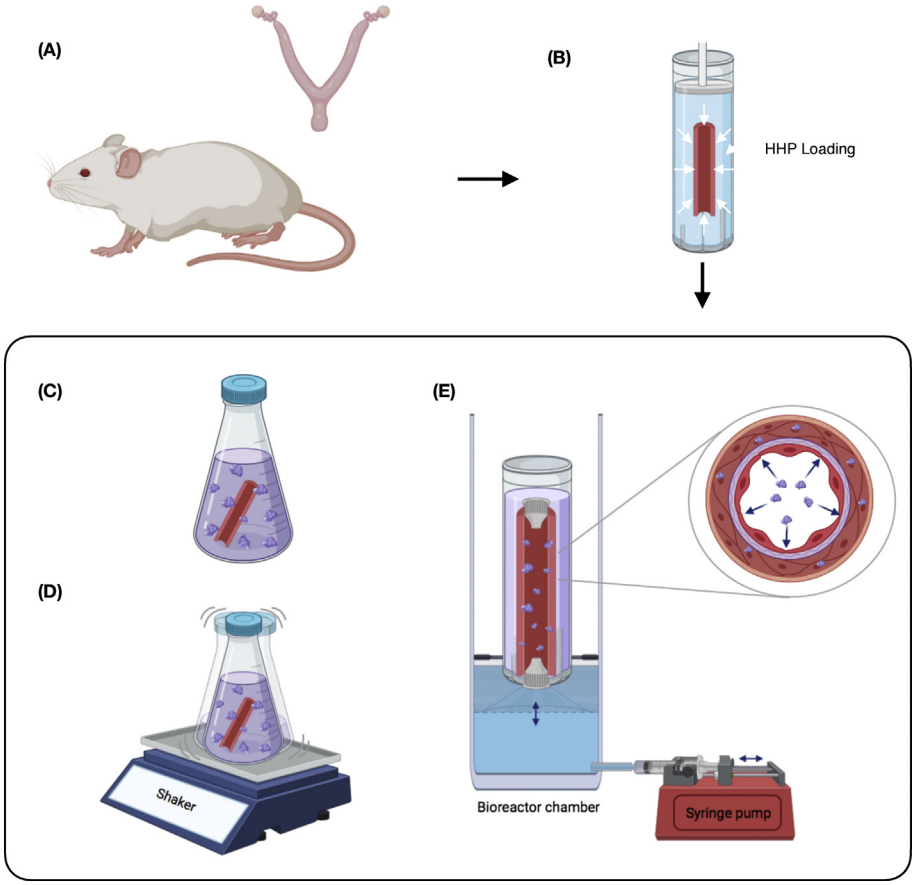


FIG. 1. Decellularization uterine tissue fabricated by high hydrostatic pressure (HHP) followed with washing process. Uterus was extracted from rat (A) prior to pressurized with high hydrostatic pressure (B). After uterine tissue was pressurized, washing methods of decellularized tissue were conducted by three different conditions: tissue washing with static condition (C), shaking condition (D) and tissue washing by using internal radial perfusion bioreactor (E). All the washing treatments were conducted in sterile incubator at 4 °C for 7 days.

on decellularized extracellular matrix on culture dish in 37 °C sterilized-cell culture incubator with 5% $CO_2$ injection and operated for 48 h (Fig. 3A).

**Perfusion system design for recellularization** The schematic diagram of internal radial perfusion bioreactor for recellularization is shown in Fig. 3B. The internal radial perfusion system for recellularization was performed by placing 25 mm length of decellularized uterine matrix on tissue frame. Both top and bottom ends were attached with 4 mm opened-end polypropylene frame stand. The tissue frame was connected to funnel cell chamber in tissue culture chamber. 5 ml of DMEM-LG with $2.0 \times 10^6$ cells/ml of rat endometrial stromal cells (approximately $1.0 \times 10^7$ cells/sample) were added into internal tissue chamber through internal lumen of uterine tissue and closed with 0.22 μm baffle on the top end of frame stand. Internal perfusion was generated through water chamber with silicone membrane by infuse/withdraw-continuous syringe pump (Legato, KD Scientific). The syringe pump was set to 0.1 Hz with 1500 μl/min infuse/withdraw rate. The bioreactor, then, was placed in 37 °C sterilized-cell culture incubator with 5% $CO_2$ injection and operated for 48 h.

**Quantification analysis of biochemical molecules** Uterine tissue samples were freeze-dried for a minimum of 12 h using a vacuum freeze dryer (FDU1200, EYELA, Tokyo, Japan). The dried samples were digested in a lysate buffer containing 446 mg/ml of papain, 5 mM cysteine-HCl and 5 mM EDTA-2 Na and incubated at 60 °C for 15 h. The samples were further refined using a homogenizer and an ultrasonic cell disruptor. DNA contents were quantified by a commercial DNA assay kit (Quant-iT PicoGreen dsDNA assay kit; Invitrogen, Waltham, MA, USA). Each sample was prepared and quantified according to the standard procedure and analyzed with a fluorospectrophotometer at a wavelength 522 nm. Dimethylmethylene Blue Assay (DMMB) was performed to quantify proteoglycan amounts. Each sample was processed with 100 μl Dimethylmethylene Blue (DMMB) in and analyzed with a fluorospectrophotometer at a wavelength 620 nm. Hydroxyproline assay were used to quantify the collagen contents of each sample. Fifty micro-liters of 4 M NaOH was mixed with 50 μl of each sample. Samples were heated in oven at 120 °C for 30 min. Then, the samples were putted on the ice cold and added with 50 μl of 1.4 N citric acid and 250 μl of chloramine-T solution and waited for 20 min for reaction. Aldehyde-perchloric acid reagent (250 μl) was added into the sample solution and incubated at 70 °C for 20 min. Each sample was analyzed with a fluorospectrophotometer at a wavelength of $\lambda = 450$ nm.

**Histological analysis of recellularized tissue** The recellularized samples were briefly rinsed with PBS and fixation was proceeded by embedding samples in 10% neutral buffered formalin solution for 8 h. Samples were dehydrated with 30% sucrose solution for minimum of 2 h and embedded in OCT compound (Tissue-Tek: 4583) and frozen in liquid nitrogen. The OCT samples were sliced into 5-μm sections with cryostat machine (Leica CM1850, Leica, Wetzlar, Germany). For histological analysis, the samples were stained with vimentin immunofluorescent and 4',6-diamidino-2-phenylindole (DAPI) staining to confirm region of endometrial stromal cells in the tissue after the recellularization. Briefly, prior to primary antibody stain, antigen retrieval was performed with 10 mM citrate buffer, under pH 6.0 at 60 °C for 1 h. Sections were blocked with protein block (X0909, Dako, Agilent Technologies, Santa Clara, CA, USA) and incubated for 8 h at 4 °C with anti-vimentin (1:400, ab92547, Abcam, Cambridge, UK) followed by Alexa Fluor 488 goat anti rabbit IgG (Invitrogen) in a room temperature for 1 h followed by 4′,6-diamidino-2-phenylindole solution (Cellstain, Dojindo, Kumamoto, Japan). Stained samples were mounted with fluorescence mounting medium (Dako). The histological structures of samples were analyzed by confocal microscope (FV30000, Olympus, Tokyo, Japan) with 405 nm and 488 nm wavelength.

**Statistical analysis** Statistical analyses were conducted using the RStudio computer software for Mac (ver. 1.4.1717). Data are presented as the mean ± S.E. T-test, Tukey–Kramer, Steel–Dwass test were used to determine the data. P value less than 0.05 were consider statistically significant.

## RESULTS

**Decellularization of uterus** The DNA contents of the decellularized tissues were quantified to evaluate the effectiveness of cell removal according to different conditions. Decellularized uterine tissue with high hydrostatic pressure followed by 7 days internal perfusion washing significantly removed 90% of DNA contents compared to native tissue. For static (control) and shaking treatments, the DNA was removed to 45% and 67%, respectively (Fig. 2B). Regarding collagen contents in the pressurized uterine tissue with the internal radial perfusion bioreactor, static and shaking treatment with high hydrostatic pressure showed no significant decrease compared to the native tissue uterine tissue. The collagen content of static, shaking and bioreactor treatment compared to native were 87%, 89% and 87%, respectively (Fig. 2C). Pressurized uterine tissue with internal radial perfusion bioreactor, static and

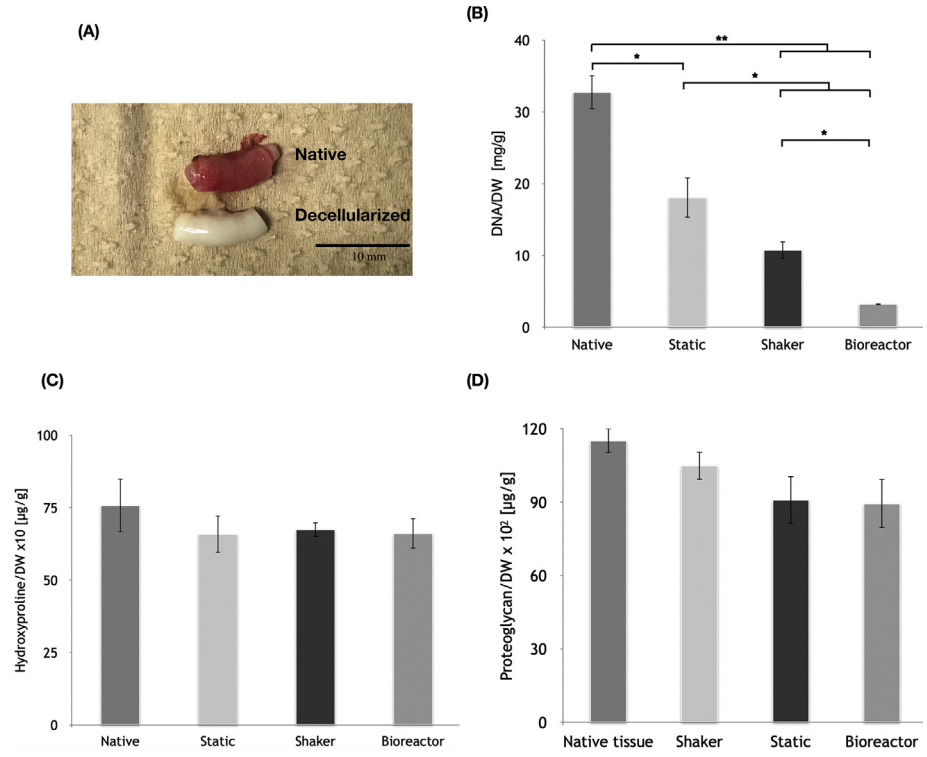


FIG. 2. Properties of decellularized tissue. Native uterine tissue compared to decellularized uterine tissue (A). Biochemical molecules were evaluated to determine scaffold properties of decellularized tissue treated with different washing process. Removal of DNA was evaluated by DNA quantification; n = 4 (B). Collagen content was evaluated by hydroxyproline quantification; n = 4 (C). Proteoglycan content was evaluated by proteoglycan quantification using DMMB assay; n = 4 (D). All the quantification assays were normalized by dry weight of tissue. Data are presented as the mean ± S.E. P values were determined using T-test, two-tailed, two-sample equal and Tukey–Kramer test; $^{*}P < 0.05$, $^{**}P < 0.005$.

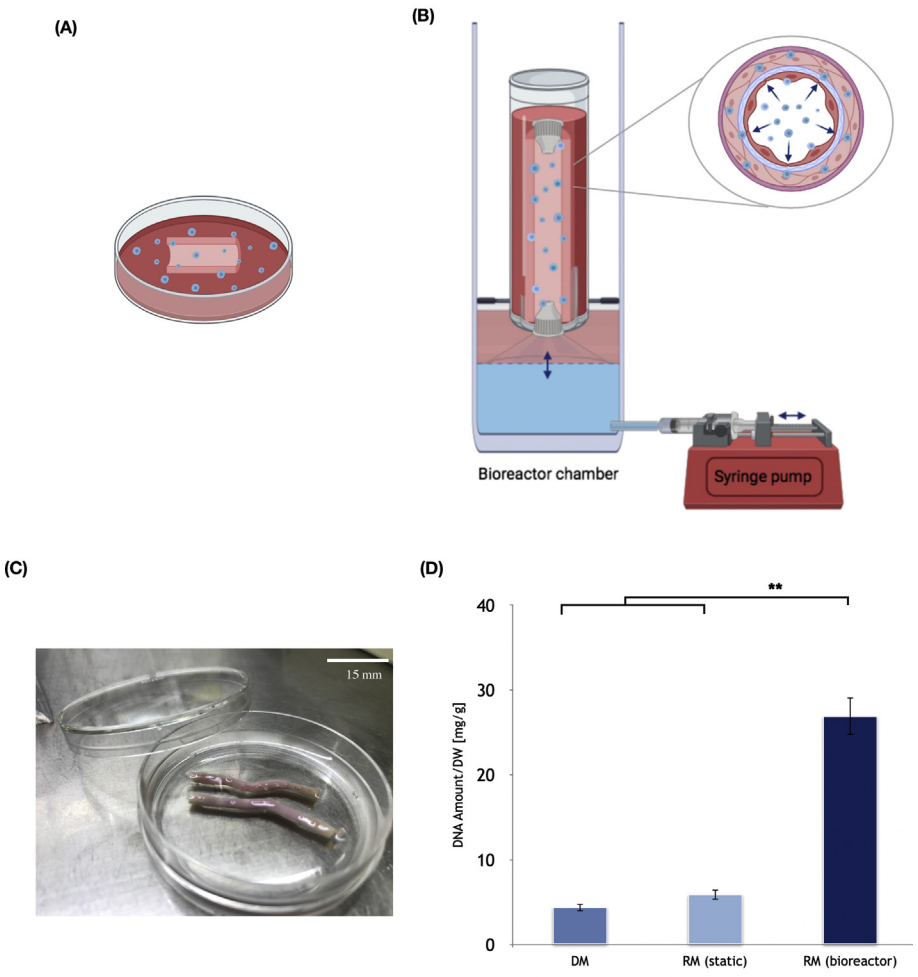


FIG. 3. Recellularization of decellularized uterine tissue. Recellularization of decellularization was performed in two different methods: conventional static cell culture on tissue (A), recellularization decellularized uterine tissue by using internal radial perfusion bioreactor (B). Both recellularization methods were conducted in sterilized-cell culture incubator with 5% injected $CO_2$ at 37 °C and operated for 48 h. Recellularized uterine tissue performed by internal radial perfusion bioreactor (C). DNA quantification was performed to evaluate DNA content of decellularized matrices (DM) and recellularized matrices (RM) performed by internal radial perfusion bioreactor; n = 6 (D). The quantification assays were normalized by dry weight of tissue. Data are presented as the mean ± S.E. P values were determined using Steel–Dwass test; $^{*}P < 0.05$, $^{**}P < 0.005$.

shaking treatment with high hydrostatic pressure showed no significant change in proteoglycan contents compared to the native tissue. The proteoglycan contents of static, shaking and internal perfusion bioreactor treatment were 89%, 77% and 76%, respectively (Fig. 2D).

**Recellularization of uterus** Recellularization was conducted by using the internal radial perfusion bioreactor compared to the conventional static cell culture and non-seeded sample. To evaluate the cell penetration of recellularized tissue, the DNA quantification and histology of uterine tissue were performed. The DNA contents of recellularized tissue was quantified by using PicoGreen assay to evaluate successful of cell seeding in uterine tissue. The results showed that the DNA content in recellularized tissue fabricated by internal radial perfusion bioreactor was significantly increased 614% compared to decellularized matrix and 457% compared to recellularized matrix with static condition (Fig. 3D). Histological analysis of DAPI staining showed the cell penetration and distribution in the endometrium and myometrium layer of the recellularized tissue. The results revealed that by using internal radial perfusion bioreactor, the cells were successfully seeded and penetrated inside the decellularized tissue as many nucleuses were observed after the recellularization. For the static culture, only few nucleuses were shown while the nucleus was not shown in the decellularized tissue (Fig. 4). Vimentin was stained to confirm the existence of endometrial stromal cells seeded in tissue. The results showed that the vimentin was strongly expressed in the bioreactor group while the expression of vimentin was not detected in the static group and decellularized tissue.

## DISCUSSION

The rapid advancement of tissue engineering and regenerative medicine has developed to challenge end-stage organ failure which orthotropic transplantation is required as an only definitive treatment for chronic end-stage disease. Decellularized matrices is a promising solution to overcome the problem of low availability and compatibility of donor organs and need for long-term immunosuppressant therapy by removing cellular immunes and antigens from tissue (7,13).

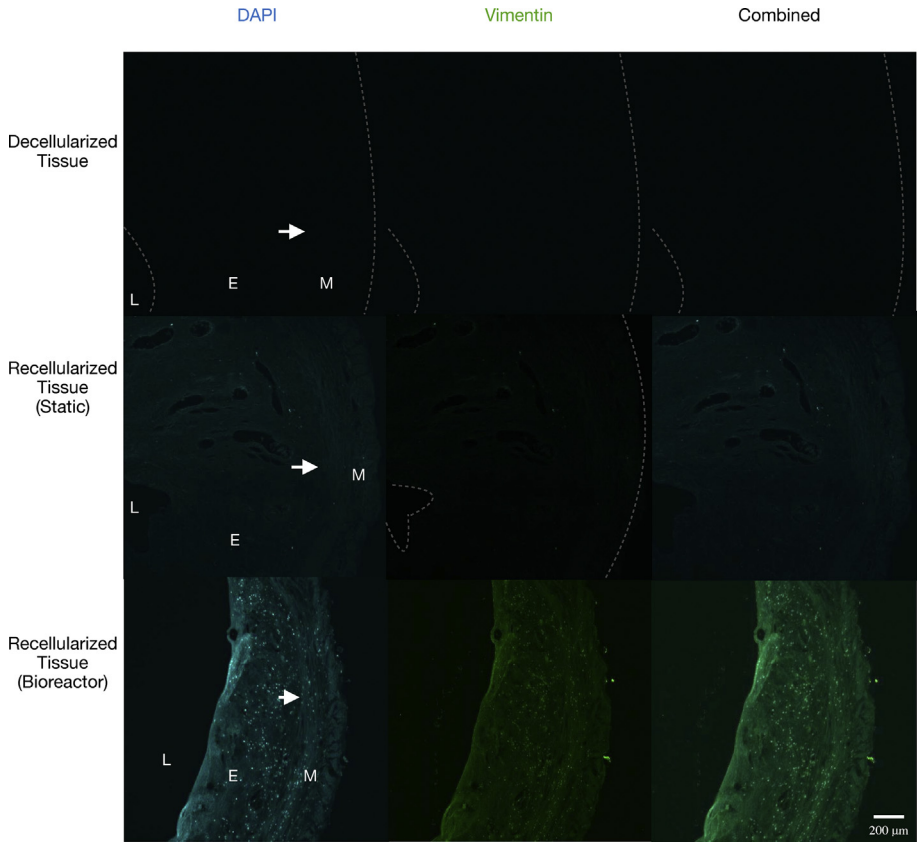


FIG. 4. DAPI/Vimentin staining of decellularized tissue, recellularized tissue with static condition and recellularized tissue by internal perfusion bioreactor. DAPI was used as a nuclear counterstain (blue) while vimentin antibody was stained to confirm region of endometrial stromal cells in the tissue (green). M, myometrium; E, endometrium; L, lumen.

The development of decellularize method for fabricate decellularized matrices is essential to promote tissue regeneration. The properties of decellularized matrices were evaluated by cell removal and presence of extracellular matrix components such as collagen and proteoglycan (7,13,18,19). In this research, we decellularized uterine tissue with high hydrostatic pressure and compared three kinds of decellularization washing methods: static, shaking and internal perfusion bioreactor.

The result from DNA quantification showed that these three washing treatments could not completely remove DNA from uterine tissue. Nonetheless, washing with internal perfusion bioreactor method could remove DNA up to 90% compared to native tissue. To improve DNA removal, other methods of cell removal should be considered such as harsher conditions, a longer treatment time or chemical treatment such as SDS, Triton X. However, such method could damage or denature extracellular matrix components which decrease the tissue regeneration properties (11,13,15–17).

Collagen and proteoglycan are the main components of extracellular matrix and promote tissue regeneration. In this research, collagen and proteoglycan were quantified to measure decellularized extracellular matrix properties as a candidate scaffold (11,19). The amount of collagen and proteoglycan of the decellularized matrix showed non-statistical different compared to the native tissue.

Previous research in uterine tissue regeneration attempted to recellularize decellularized uterine tissue by cell injection, static culture and perfusion through artery (15–17). However, those research were not successful in achieving cell seeding inside of decellularized uterine tissue. For static and injection methods cells were able to seed only on the surface of the tissue while cells were not observed inside tissue (15,16,20). For perfusion recellularization, cells were partially observed on epithelium layer of uterine tissue, but were not observed in endometrium and myometrium layer (17). This indicates that the recellularization was promoted by using perfusion system, compared to static culture. In this research, we newly developed the internal radial perfusion system to promote recellularization in all layers of uterine tissue. As a result of recellularization by recruiting the internal radial perfusion, the recellularization was successfully achieved. With the internal perfusion bioreactor, it helped to penetrate the solution inside the tissue cavity to the myometrium during decellularization and recellularization process. Moreover, due to the end-close system, the pressure was generated in radical direction during the infuse stage which improved washing buffer solution and cell suspension to penetrate through tissue membrane radically, as shown in Fig. 4, the cells were observed inside the tissue due to the penetration by the generated radial pressure. However, to proceed better decellularization and recellularization, the optimum condition might be investigated in further research. With the achievement of recellularization of uterine tissue by internal radial perfusion bioreactor, this engineered tissue is promising to be utilized for promote cell proliferation and tissue regeneration after the engineered tissue is implanted.

## ACKNOWLEDGMENTS

This work was financially supported by Grant-in-aid for Scientific Research A (19H01173, 21H05775, 21K19889, 19H01173), ASTEP, The Precise Measurement Technology Promotion Foundation and Amada Foundation in Japan. The authors declare no conflicts of interest.

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