Fabrication of uterine decellularized matrix using high hydrostatic pressure through depolymerization of actin filaments

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Abstract

Recently, many groups in the field of tissue engineering have attempted to utilize decellularized matrices for tissue regeneration. The decellularized matrices are known as a suitable tissue-engineered scaffold that retains the original structure of extracellular matrix (ECM) in the native tissue as well as its complex vasculature. While chemical reagents such as sodium dodecyl sulfate (SDS) are generally selected to fabricate the decellularized matrices due to its ease of use, high hydrostatic pressure (HHP) has become a powerful alternative to induce the decellularization without using any chemical reagents which have a possibility to provoke inflammatory response by the residual chemicals after in vivo transplantation. Although the HHP has been regarded as a promising tool to decellularize the native tissue, its fabrication mechanism remains still unknown. The aim of this study was to investigate the fabrication mechanism using HHP of 980 MPa to decellularize uterine tissues harvested from Sprague Dawley rats. As a result of histochemical analysis, we first reported that actin filaments in the uterine tissue were depolymerized by applying HHP. Our present findings will lead to the optimization of fabrication method using hydrostatic pressure to have an optimal decellularize matrix with complete micro- and macro-structures of the native tissue for tissue regeneration.

Keywords: Decellularization, Scaffold, Uterus, Hydrostatic pressure, Uterine regeneration

1. Introduction

The aim of tissue engineering is to regenerate or replace damaged tissues by using cells with a combination of scaffold, biochimical, or mechanical factors (Ting et al., 2015; Kim et al., 2017; Vacanti and Langer 1999; Howard et al., 2008; Asaoka et al., 2013). Among those factors, the scaffold is regarded as an essential factor for cells to adhere and develop into tissues in vitro by providing the structural support (Chan and Leong 2008). Several requirements should be considered to become a candidate of the scaffold, such as biocompatibility, biodegradability, porosity, or mechanical properties (O'Brien 2011). In order to encounter those requirements, many researchers have developed and utilized numerous types of scaffolds made of synthetic or natural polymers (Asti and Gioglio 2014; Andy T H Wu et al., 2015).

For the last couple of decades, use of decellularization matrix (DM) has also been highlighted for scaffolding in the regenerative medicine and applied for various types of organ model such as heart (Ott et al., 2008), kidney (Song et al., 2013), bladder (Rosario et al., 2008), cartilage (Kheir et al., 2011), uterus (Miyazaki and Maruyama 2014; Hiraoka et al., 2016), etc. This biologic scaffold derived from extracellular matrix (ECM) preserves its native architecture and further
exerts a low host immune response (Crapo et al., 2011). Chemical agents such as sodium dodecyl sulfate (SDS) or triton X-100 are widely used for the decellularizing process, but it has a potential risk of residual toxicity in the host tissue when the DM is transplanted (Gilbert et al., 2006).

As an alternative agent to induce the decellularizing process, a high-hydrostatic pressure (HHP) method has been reported. Applying the HHP of 980 MPa successfully decellularized both soft and hard tissue models including cornea (Hashimoto et al., 2010), blood vessel (Funamoto et al., 2010), and bone (Hashimoto et al., 2011). Notably, our group first reported the application of uterine DM fabricated by HHP using murine model for partial uterine regeneration (Santoso et al., 2014). While our group has worked on several studies elucidating the effect of hydrostatic pressure on various cell models (Montagne et al., 2017; Kim et al., 2014; Kawanishi et al., 2007), the fabrication mechanism of decellularization process using HHP remained still unknown. By understanding this mechanism, it enables to lead to a new strategy developing an effective process of the decellularization or understanding the effect of HHP in the native tissue.

In this study, we attempted to elucidate the fabrication mechanism of decellularization process using uterine tissues under HHP. After creating the uterine DM by HHP, we carried out various histological staining and immunostainings to observe the histological change induced by hydrostatic pressures or washing buffer in the native uterine tissue.

2. Materials and methods

2.1 Uterine tissue sample preparation

In this study, we used 9 weeks old female Sprague Dawley (SD) rats purchased from CLEA Japan, Inc. The uterine horns were isolated from the rats in Fig.1 (A) and rinsed with phosphate buffered saline (PBS) to remove the blood. As performed in the previous study (Santoso et al., 2014), the uterine horns were subjected to an incision along the mesometrium line and cut into 15 mm long for decellularization as shown Fig.1 (C).

2.2 High-hydrostatic pressure

In this study, we prepared the decellularized samples by applying high-hydrostatic pressure (HHP) followed by washing process. The native uterine tissues were first packed in a plastic bag filled with PBS and pressurized using a cold isostatic pressurization machine (Dr. Chef; Kobelco, Japan). As in the previous study (Santoso et al., 2014), the chamber inside the machine was pressurized up to 50 MPa or 980 MPa at 65.3 MPa/min. After the pressure was maintained for 10 minutes, it reduced to the atmospheric pressure at 65.3 MPa/min as described in Fig.1 (B). The temperature in the chamber was kept at 30 °C. The HHP samples were collected for analysis or treatment with washing buffer.

2.3 Washing process

After the HHP treatment, the sample were treated with a washing buffer containing 0.2 mg/ml DNase I (Roche, USA), 0.9% NaCl (Wako, Japan), 0.05 M magnesium chloride hexahydrate (Wako, Japan), and 1% penicillin and streptomycin (Gibco, Japan). The washing process was performed on a shaker at 4 °C for 7 days.

2.4 DNA assay

The samples were freeze-dried overnight by using a freeze drier system (EYELA, Japan) and measured its dry weight. The samples were then lysed using a papain buffer containing 446 μg/ml papain, 5 mM cysteine-HCl and 5 mM EDTA-2Na at 60 °C for 16 hours. The lysed samples were treated with a power homogenizer (Polytron PT3100, Kinematica, Switzerland) and an ultrasonic cell disruptor (XL-2000 Microson, Labcare Systems, UK). By using a Quant-iT PicoGreen dsDNA Reagent kit (Thermo, USA), the amount of DNA in the sample was measured spectrophotometrically with an EnSpire microplate reader (PerkinElmer, USA) at an absorbance wavelength of 522 nm.

2.5 Histochemical assay

The samples were rinsed with PBS and fixed in 10% formalin neutral buffer solution (Wako, Japan). All the samples were embedded into O.C.T. compound (Sakura Tissue-TEK, Japan) and stored as the frozen block. The sections of the
sample were prepared at 5 μm thickness by a cryostat. The O.C.T. compound in the frozen block was removed through rinse with water for 5 min prior to histological stains. According to the manufacturer’s protocol, hematoxylin and eosin (Sigma, USA), and Masson’s Trichrome (Muto, Japan) were carried out. For immunostaining, we utilized DAPI (Thermo Fisher Scientific, USA) and Actin-stain™ 555 Fluorescent Phalloidin (Cytoskeleton, Inc., USA) to stain cell nucleus and actin filaments (F-actin), respectively. Phosphorylated H2AX (γH2AX) (Abcam) was also stained for a marker of DNA damage response.

2.6 Statistical analysis
All the bars of data represent the means ± standard error. Statistic analysis of data was carried out by a student’s t-test in order to assess the statistical significance. A p-value of 0.05 or less was assumed to become statistically significant.

3. Results
3.1 Fabrication of uterine decellularized matrix using high hydrostatic pressure
Uterine horns were isolated from SD rats and then treated with HHP (980MPa) as described in Fig.1 (B). After treating with HHP followed by washing buffer, a color of the uterine tissue became whiter in Fig.1 (C) – (E). In Fig. 1(F), the sample subjected to HHP and washing buffer was evaluated by DNA assay. Its DNA content was tremendously declined to 5% (p < 0.005) compared to the native tissue. Therefore, applying HHP followed by washing buffer successfully removed the DNA contents in the tissue.

Fig. 1 Uterine decellularized matrix fabricated by high hydrostatic pressure. (A) Native tissue isolated from Sprague Dawley rat. (B) Pressure-time graph during pressurizing the sample. (C) Native rat uterus tissue. (D) Tissue immediate after loading high hydrostatic pressure. (E) Tissue treated with high hydrostatic pressure followed by washing process for 7 days. (F) DNA contents normalized to dry weight in the native tissue (n = 4) and the tissue treated with high hydrostatic pressure and washing process (n = 4). Values represent the mean of n = 4 independent biological replicates (p-value was obtained from student’s t-test; ** p < 0.005).
3.2 Evaluation of histological change in decellularized matrix using metachromatic staining

In Fig.2 (A) – (C), hematoxylin and eosin (HE) staining images showed that the sample treated only by HHP kept almost same amounts of cells in the entire tissue as native tissue. It, however, had few cells left inside the tissue after treating with HHP and WB. Masson’s trichrome (MT) staining was then carried out to distinguish the collagen fiber.

Fig. 2 Metachromatic staining images. Hematoxylin and eosin (HE) staining images of (A) native tissue, (B) the tissue after applying high hydrostatic pressurization (HHP), and (C) the tissue treated with HHP and washing process for 7 days. Masson’s trichrome (MT) stain images of (D) native tissue, (E) the tissue after applying HHP, and (F) the tissue treated with HHP and washing process for 7 days. e, endometrium (inner layer of the uterus); g, uterus glands; m, myometrium (outer layer of the uterus). The staining was independently repeated nine times. White and black bar represent 200 μm and 400 μm, respectively.

In Fig.2 (A) – (C), hematoxylin and eosin (HE) staining images showed that the sample treated only by HHP kept almost same amounts of cells in the entire tissue as native tissue. It, however, had few cells left inside the tissue after treating with HHP and WB. Masson’s trichrome (MT) staining was then carried out to distinguish the collagen fiber.

Fig.3 Immunofluorescence staining images. DAPI staining of (A) native tissue, (B) the tissue after applying high hydrostatic pressurization (HHP), and (C) the tissue treated with HHP and washing process for 7 days. Phosphorylated H2AX (γH2AX) staining of (D) native tissue, (E) the tissue after applying high hydrostatic pressurization (HHP), and (F) the tissue treated with HHP and washing process for 7 days. Actin stain (G) native tissue, (H) the tissue after applying high hydrostatic pressurization (HHP), and (I) the tissue treated with HHP and washing process for 7 days. The staining was independently repeated four times. The white bar represents 50 μm.
(blue) and keratin or smooth muscle fiber (purple) in the tissue. While the native tissue had the strong purple part stained in the inner layer of the endometrium, myometrium, and around the uterine glands in the uterine tissue, the strong purple stained parts were remarkably weakened in the HHP and HHP+WB sample (Fig.2 (D)-(F)). Despite the removal of the purple part in the HHP treated samples, the collagen part (blue) was preserved after applying HHP or HHP followed by washing process.

3.3 Evaluation of histological change in decellularized matrix using immunofluorescence

We then performed immunofluorescence to observe any change in actin filaments (F-Actin) and γH2AX before and after HHP or WB treatment. Fig.3 (A) – (C) depicted that the nucleus stained by DAPI seemed to be similar before and after loading HHP into the native tissue while there are few nuclei left in the HHP + WB sample. Moreover, the DAPI staining in Fig.3 (A) and (B) indicated that the ellipse shape of nucleus shown in the native tissue became deformed immediate after HHP. In Fig. 3 (D) – (F), γH2AX was strongly expressed in the HHP sample while there was no expression shown in the native tissue or HHP + WB sample. As shown in Fig.3 (G), F-Actin was strongly stained along the inner layer of endometrium, myometrium, and around uterine glands in the native tissue. However, application of HHP fully depolymerized the F-Actin in the HHP samples as described in Fig.3 (H) and (I).

3.4 Evaluation of histological changes after 50 MPa pressurizing

To elucidate the effect of hydrostatic pressure on the native tissue, we also applied a constant 50 MPa pressure to the native uterine tissue. As a result of HE, DAPI, or γH2AX staining in Fig. 4, the pressure of 50 MPa did not significantly deform cell nucleus in the tissue. On the other hand, MT or F-Actin staining images showed that the 50 MPa pressure did not induce depolymerization of the actin filaments in the native tissue.
3.5 Histological changes of pressurized tissue by washing process

In order to check the role of WB, all samples including native tissue or tissue applied by 50 or 980 MPa were subjected to the washing process for 7 days. In Fig. 5 (A) – (D), HE staining results showed that washing buffer alone could not remove the cells inside the native tissue and the tissue pressurized by 50 MPa. MT staining results in Fig.5 (E) – (H) also indicate that the treating washing buffer alone did not eliminate the inner layer of endometrium, the outer layer of the uterine tissue, and around the uterine glands, strongly stained purple.

4. Discussion

Decellularization has been widely studied and applied in the field of tissue engineering over the last two decades. While chemical treatments such as ionic detergent including sodium dodecyl sulfate (SDS) are generally selected in the decellularization process due to the easiness of use, it may induce the residual toxicity in the host tissue when the DM is transplanted. Since the application of HHP in the decellularization was first introduced, the DM fabricated by HHP exerted its advantage avoiding the use of toxic chemical agents. Although our group first attempted to develop the uterine DM fabricated by HHP and successfully transplanted it in the murine models for partial uterine regeneration, the fabrication mechanism of DM using HHP remained unknown.

As in the previous study, we successfully fabricated the uterine DM using HHP followed by washing process. The uterine tissue collected from a SD rat was subjected to a hydrostatic pressure of 980 MPa for 10 minutes and then treated with a washing buffer for 7 days. From the results of DNA quantification and several staining images, we showed that most of the residual cells after applying the HHP process were removed followed by the washing process, while it retained its native structure.

In this study, we first reported that the HHP depolymerized the actin filaments in the uterine tissue as shown in Fig.2 and Fig.3. In the MT staining, the keratin/muscle fibers and collagen fibers are stained purple and blue, respectively (Darios et al., 2012). By applying the HHP to the native uterine tissues, the eliminated parts stained purple in the MT staining corresponded to the F-actin in the native tissue while their nucleus were still remained immediate after HHP. By depolymerizing the actin cytoskeleton in the native tissue, it may provide pathways for the pressurized cells to be washed away during the washing treatment for 7 days. Although the HHP alone could not remove the cells, the nucleus inside the tissue shown in the DAPI staining image was deformed by applying HHP as the DNA damage marker, γH2AX, was strongly expressed in the deformed nucleus.

In order to highlight the significance of HHP, we performed experiments treating native tissues or tissue pressurized
by 50 MPa with washing process. Although the HHP (980 MPa) has been used to decellularize various types of the organs, it may be so excessive that it has a risk to damage its native structure. In this study, we investigated if the pressure of 50 MPa can induce the decellularization process. Eventually, the results in Fig.4 and Fig.5 represented that 7 days of washing treatment against the native tissue or the tissue pressurized by 50 MPa failed to remove the cells inside the tissue as well as their actin cytoskeleton. Moreover, we confirmed that the role of washing buffer was not to induce the cell deformation, but it was to wash away the damaged cells. Applying 50 MPa did not also induce significant cell deformations or removal of the actin cytoskeleton.

For several decades, effect of mechanical stimuli on various cell models has been widely studied (Kim et al., 2017; Montagne et al., 2014; Furukawa et al., 2000). There are several studies on the effect of hydrostatic pressure upon actin cytoskeletons, reporting that the cell structure is regulated by hydrostatic pressure (Wu et al., 2008; Tokuda et al., 2009; Garcia et al., 1992). In Garcia’s study, F-actin in rat skeletal muscle was dissociated under a hydrostatic pressure of 240 MP. This report supports the result of the F-actin depolymerization in the uterine native tissue by 980 MPa as represented in Fig. 3. While applying excessively high value of hydrostatic pressure, 980MP, depolymerized the actin cytoskeleton in the uterine tissue, we observed that the HHP damaged and deformed the shape of cell nucleus inside the tissue. However, requirements for removal of actin cytoskeletons during the decellularization still remain unknown. Moreover, the excessive HHP has a risk to damage the original structure of the native tissue although it enables to decellularize the tissue, so that the optimization of HHP will be required in the further study. The desired intensity for the pressure to induce the decellularization while retaining the native structure needs to be addressed according to different types of tissues, organs, or animals since they have different mechanical properties and cell/extracellular matrix components. In the future, this desired pressurizing condition can be achieved by finding out the minimum pressure that enables to induce the decellularization process with removal of actin cytoskeleton.

5. Conclusion

We successfully fabricated the uterine decellularization matrix using HHP. Our present finding indicated that the HHP of 980 MPa depolymerized the actin cytoskeletons in the native rat uterine tissue during decellularization process. We also observed that it altered the nuclear morphology immediate after applying the HHP to the uterine tissue. When exploring further study, these results may lead to a full understanding for the fabrication mechanism of the decellularization by HHP. By understanding this fabrication mechanism, it contributes to development of a novel method to reconstruct a biologic scaffold derived from native ECM with complete native micro- and macro- structures using other biochemical or mechanical force.

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