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Towards the development of a bioengineered uterus: Comparison of different protocols for rat uterus decellularization

M. Hellström [a,*], R.R. El-Akouri [a], C. Sihlbom [b], B.M. Olsson [b], J. Lengqvist [b], H. Bäckdahl [e], B.R. Johansson [c], M. Olausson [d], S. Sumitran-Holgersson [d], M. Brännström [a]

[a] Laboratory for Transplantation and Regenerative Medicine, Institute of Clinical Sciences, Department of Obstetrics and Gynecology, Sahlgrenska Academy, University of Gothenburg, Sweden
[b] Proteomics Core Facility, University of Gothenburg, Gothenburg, Sweden
[c] Electron Microscopy Unit, Institute of Biomedicine, University of Gothenburg, Gothenburg, Sweden
[d] Department of Surgery, Institute of Clinical Sciences, Sahlgrenska Academy, University of Gothenburg, Sweden
[e] Chemistry Materials and Surfaces, SP Technical Research Institute of Sweden, Borås, Sweden

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ABSTRACT

Uterus transplantation (UTx) may be the only possible curative treatment for absolute uterine factor infertility, which affects 1 in every 500 females of fertile age. We recently presented the 6-month results from the first clinical UTx trial, describing nine live-donor procedures. This routine involves complicated surgery and requires potentially harmful immune suppression to prevent rejection. However, tissue engineering applications using biomaterials and stem cells may replace the need for a live donor, and could prevent the required immunosuppressive treatment. To investigate the basic aspects of this, we developed a novel whole-uterus scaffold design for uterus tissue engineering experiments in the rat. Decellularization was achieved by perfusion of detergents and ionic solutions. The remaining matrix and its biochemical and mechanical properties were quantitatively compared from using three different protocols. The constructs were further compared with native uterus tissue composition. Perfusion with Triton X-100/dimethyl sulfoxide/H_2O led to a compact, weaker scaffold that showed evidence of a compromised matrix organization. Sodium deoxycholate/dH_2O perfusion gave rise to a porous scaffold that structurally and mechanically resembled native uterus better. An innovative combination of two proteomic analyses revealed higher fibronectin and versican content in these porous scaffolds, which may explain the improved scaffold organization. Together with other important protocol-dependent differences, our results can contribute to the development of improved decellularization protocols for assorted organs. Furthermore, our study shows the first available data on decellularized whole uterus, and creates new opportunities for numerous in vitro and in vivo whole-uterus tissue engineering applications.
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1. Introduction

Infertility affects ~15% of all couples and in most cases this infertility can be circumvented by assisted reproductive techniques (ARTs) such as in vitro fertilization. Despite the great advances in ART, one major subgroup of female infertility remains untreatable. This absolute uterine factor infertility (AUFI) is due to the absence of a functional uterus [1]. The only option for these women to gain genetic motherhood is with the assistance of a gestational surrogate mother. However, this procedure is not approved in most parts of the world for ethical, religious or legal reasons. Uterus transplantation (UTx) has been proposed as a possible curative treatment for AUFI [2], and so far 11 human UTx attempts have been made, with the majority of cases arising from a recent clinical study from our group [3]. However, no viable pregnancy has yet been reported.

Although, human allogeneic UTx may prove to be successful in terms of pregnancy, there will still be problems related to the surgical risk to retrieve the uterus from a live donor and the adverse effects that are related to the intake of immunosuppression [4]. Recent progress in stem cell research and biomaterials has given hope to the replacement of a live donor as an organ source, with a customized organ construct for transplantation using autologous cells [5], thus circumventing main obstacles in organ transplantation such as lack of donor organs and long-term immunosuppressive treatments. Successful animal studies have shown that

* Corresponding author. Tel.: +46 721 875141; fax: +46 31 7411701.
E-mail address: mats.hellstrom@gu.se (M. Hellström).

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complicated organs such as the heart [6], the liver [7], the lung [8,9] and the kidney [10] can be decellularized and recellularized with new cells to construct a tissue engineered organ with at least partial organ specific functionality. The less complex, hollow tissues were recently reconstructed for the clinic; decellularized trachea [11] and blood vessel [12] were recellularized with autologous stem cells and transplanted to patients with success in terms of restored function.

Various techniques for decellularization and recellularization have been examined for pre-clinical interventions of miscellaneous tissues, e.g., small intestine [13,14], urinary bladder [15], heart valves [16], larynx [17], dermis [18], musculofascial tissue [19] and nerves [20,21]. Concerning the female internal genital tract, small patches of myometrial tissue were recently decellularized using a modified protocol that had been optimized for decellularization of blood vessels [22]. Other tissue engineering approaches for uterine tissue include the use of scaffolds of collagen/matrigel [23–25] or silk/collagen [26], as well as scaffolds made from blood clots [27]. Promising results using biodegradable polymer scaffolds for "subtotal uterine tissue replacement" in a rabbit model was recently reported in a review [28]. It should be pointed out that the above-mentioned uterus-related tissue engineering studies were not aimed at constructing a complete organ that could replace a uterine graft at UTx. Furthermore, decellularized scaffolds created from whole organ vascular perfusion protocols provide several advantages over decellularized tissue patches or artificial scaffolds: (a) it comprises the correct tissue-specific extracellular matrix (ECM) composition, (b) it has the physical three-dimensional (3-D) appearance of the original organ with the correct conduit architecture for blood vessels and glandular ducts, (c) cells can be reintroduced and sustained in a bioreactor via the vascular conduits for ex vivo organ regeneration, (d) the ECM can be remodeled by repopulated cells and (e) the construct provides guiding cues for tissue-specific cell differentiation and migration [29–31].

The present study aimed to develop a scaffold-design standard for whole uterus which later can be used for recellularization and transplantation studies in rodents. Specifically, we compared three different protocols: two were based on the detergent Triton X-100 and the high ionic solution dimethyl sulfoxide (DMSO) as cell membrane disrupting agents; the third protocol was based on sodium deoxycholate (SDC), which disrupts cells by both detergent and ionic mechanisms. These scaffolds may also become valuable for novel uterine-specific 3-D cell culturing systems to study early embryo implantation and development and endometrium cancer cell behavior or for various in vitro drug screening applications.

2. Materials and methods

2.1. Animal work and uterus isolation

In total 35 female Lewis rats (140–180 g; Charles River, Sulzfelt, Germany) were used as whole-uterus donors. Eight of these organs were used as a comparative group for normal tissue, and the remaining 27 uteri were divided into three groups ($n = 9$ per group) and were exposed to various decellularization protocols. All animal work was approved by the Animal Ethics Committee in Gothenburg, Sweden.

The uterus isolation surgery was performed aseptically under isoflurane anesthesia. A laparotomy was performed via a mid-line incision from the symphysis pubis to the xiphoid process. To gain full exposure to the uterus and its vascular tree, the small intestines were initially retracted to the left side of the animal and packed in moistened gauze. The inferior mesenteric artery was cauterized using bipolar diathermy (Coa-Comp Bikoagulator; Instrumenta AB, Billdal, Sweden) at a maximum effect of 2 W. The colon was divided just above the rectum and retracted to the side to obtain a free operating field. In the subsequent order, the inferior epigastric vessels, the external iliac vessels and the external pudendal vessels were double ligated and cut. All vessels supplying the lower portion of the cervix and the bladder (superior vesical, inferior vesical, cervical and vaginal vessels) were then closed to prevent bleeding when the cervix was transected. Special caution was taken to avoid the vascular branches from the hypogastric trunk on both sides. The cervix was gently lifted up to expose the intact uterine artery and veins, which also enabled the attachment of a titanium clip (Weck Closure Systems Ltd, Resarch Triangle Park, NC, USA) on the superior gluteal vessels. The common iliac vessels and the lumbar vessels (2–4) were then transected between double ligatures. Both uterine horns were then double ligated and cut at a site between the tip of the uterine horn and the oviduct. The uterine horns were then dissected free from their attachments to the dorsal peritoneum and folded over to expose the hypogastric trunk. The aorta and the vena cava were tied off inferior to the ovarian vessels by 8–0 suture to create a complete segment that included the inferior aorta, the inferior vena cava and the intact vascular connection to both uterine horns. Small incisions were made in the vena cava and in the aorta so that the specimen could be gently flushed with 1–2 ml ice-cold Perfadex (+4 °C) solution (Vitrolife Sweden AB, Kungsbacka, Sweden) supplemented with xylocaine (0.4 mg ml^{-1}; Astra Zeneca, Mölndal, Sweden) and heparin (50 IU ml^{-1}; Leo Pharma AB, Malmö, Sweden). The perfusion of the isolated donor uterus was assessed successful when the uterus blanched and clear fluid excited the incision made in the vena cava. The uterus was then placed in the same solution and frozen gradually by first placing it in −20 °C, and then in −80 °C for long-term storage.

2.2. Decellularization of whole uterus and sterility control test

The uterus was thawed and the tip of a catheter prefilled with phosphate buffer saline (PBS; pH 7.4) including 0.05% sodium azide (PBS+A) was inserted into the lumen of the aorta and fixed with a ligature. A custom-made decellularization perfusion set-up was built using sterile tissue culture plastics (Supplementary Fig. S.1A) and was connected to a Masterflex perfusion pump (Cole-Parmer instruments, Chicago, USA). The uterus was then perfused with PBS+A for 30 min to remove blood remnants and cell debris. Perfusion speed was set to 6 ml min^{-1}, and the flow was divided into three 23G needle exits, where only one of them was connected to the uterine tissue (Supplementary Fig. S.1B). The remaining two open exits acted as pressure valves, to reduce the risk of possible hypertension within the uterine vasculature during perfusion. 27 uteri were randomly allocated to either of three different protocols for decellularization ($n = 9$ per group), and the decellularized constructs were compared to native uterus tissue ($n = 8$).

2.2.1. Protocol 1

(P1; $n = 9$): the uterus was perfused for 4 h with DMSO (4% in PBS+A) followed by another 4 h perfusion with Triton X-100 (1%, diluted in PBS+A), followed by 30 min perfusion with PBS+A. The uterus was then left in a PBS+A solution overnight at room temperature. The DMSO, Triton X-100 and washing steps were then repeated another four times (for a total five cycles; 5 days).

2.2.2. Protocol 2

(P2; $n = 9$): the uterus was perfused with five identical cycles as for protocol 1 but with modifications between cycles; all dilutions (including washes and overnight storage) were in dH_2O + sodium azide (0.05%; dH_2O+A) and a freeze–thaw procedure (−80 °C) was introduced between cycles 2 and 3.

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