A Combination of Physical and Chemical Treatments Is More Effective in The Preparation of Acellular Uterine Scaffolds

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Abstract

Objective: Decellularized uterine scaffold, as a new achievement in tissue engineering, enables recellularization and regeneration of uterine tissues and supports pregnancy in a fashion comparable to the intact uterus. The acellular methods are methods preferred in many respects due to their similarity to normal tissue, so it is necessary to try to introduce an acellularization protocol with minimum disadvantages and maximum advantages. Therefore, this study aimed to compare different protocols to achieve the optimal uterus decellularization method for future *in vitro* and *in vivo* bioengineering experiments.

Materials and Methods: In this experimental study, rat uteri were decellularized by four different protocols (P) using sodium dodecyl sulfate (SDS), with different doses and time incubations (P1 and P2), SDS/Triton-X100 sequentially (P3), and a combination of physical (freeze/thaw) and chemical reagents (SDS/Triton X-100). The scaffolds were examined by histopathological staining, DNA quantification, MTT assay, blood compatibility assay, FESEM, and mechanical studies.

Results: Histology assessment showed that only in P4, cell residues were completely removed. Masson's trichrome staining demonstrated that in P3, collagen fibers were decreased; however, no damage was observed in the collagen bundles using other protocols. In indirect MTT assays, cell viabilities achieved by all used protocols were significantly higher than the native samples. The percentage of red blood cell (RBC) hemolysis in the presence of prepared scaffolds from all 4 protocols was less than 2%. The mechanical properties of none of the obtained scaffolds were significantly different from the native sample except for P3.

Conclusion: Uteri decellularized with a combination of physical and chemical treatments (P4) was the most favorable treatment in our study with the complete removal of cell residue, preservation of the three-dimensional structure, complete removal of detergents, and preservation of the mechanical property of the scaffolds.

Keywords: Acellularization, Female Infertility, Rat Uterus, Sodium Dodecyl Sulfate, Tissue Engineering

Introduction

Absolute uterine factor infertility (AUFI) is one of the infertility causes, which refers to the absence or dysfunction of the uterus and has a prevalence of one in every 500 women of fertile age (1). Currently, the only solution available is gestational surrogacy, which comes with its own set of economic, legal, and ethical challenges (2). While organ transplantation has been proposed as a solution, due to the scarce availability of human organs and immunogenic effects, many investigators have been directed towards the utilization or development of bioengineered tissue by a synthetic mold or acellularized scaffold to restore fertility. This approach takes advantage of the body's inherent ability to regenerate organs, and repairs the defect of reproductive organs with a combination of cells, growth factors, and biological materials with repairing (3). So far, many studies have used biodegradable polymer scaffolds [such as poly-DL-lactide-coglycolide (PLGA)-coated polyglycolic acid

(PGA) (4)], collagen/matrigel (5), silk-coated collagen (6), or boiled blood clots (7). Despite the excellent mechanical support and stability of synthetic scaffolds, decellularized extracellular matrix (ECM) of the target tissue is one of the preferred methods of engineered tissue. The main benefit of this method is the minimized immune system reactions (due to the removal of membrane antigen epitopes and intracellular derivatives) as well as functional, mechanical, and structural similarities to the native tissues (8).

The use of decellularized scaffolds for the engineering of vital organs such as kidneys (9), heart (whole organ) (10), blood vessels (whole organ) (11), and bone (12) have been reported. This technique already has been transferred to clinical trials (13).

Several methods have been tested to remove cells from the ECM (14-17). Physical treatments such as freeze-thawing procedures, sonication, agitation, mechanical

pressure, and enzymatic treatment (using dispase, trypsin, esterases, or nucleases) have also been employed to remove cells from tissues (14, 18). Due to the diversity of cell morphology, matrix thickness, and variable density from organ to organ, the optimal acellularization method is unique to each tissue (17). The methods used to produce uterine tissue ECM scaffolds show distinct effects on the structural and functional components of the scaffold material preparation, some of which even have adverse effects on immunological, structural, and mechanical properties (19). Therefore, introducing an efficient decellularization protocol for tissue engineering of the uterine is very necessary.

Detergents are effective in removing nuclear remnants and cytoplasmic proteins from tissues, but their optimum dose and exposure time are important for tissue properties. White et al. (20) showed increasing sodium dodecyl sulfate (SDS) concentrations from 0.1% to 1.0% increased both the intensity of SDS fragments and adverse cell outcomes. Most studies have used detergents to remove cells from uterine tissue, and there are fewer reports of combining these methods with other methods, such as mechanical cell removal, so we developed four decellularisation protocols, In the first three protocols, only detergent (chemical) was used, but in the fourth, a combination of physical (freeze/thaw) and chemical reagents was used. Therefore, we evaluated the decellularization efficacy of each method using different methods such as H&E which evaluates cell component removal, mechanical testing for ECM structure preservation, MTT for detergent removal, and so on. We aimed to determine which method was advantageous in cell removal and preserving the ECM components, structure, and mechanical properties of natural uterine for an ideal scaffold for tissue engineering.

Materials and Methods

Animals

All animals used in this experimental study were housed in the animal house facility according to the institutional ethical guidelines for using laboratory animals.

Fifty rats were randomly divided into five groups (n=10 per group). Female Wistar rats were put down, 9-16 weeks old and weighing 180-200 g, in an estrous cycle (which was characterized by the presence of abundant anucleated cornified epithelial cells in the cytology of vaginal smear) (21). Rats were anesthetized with an injection of ketamine (70 mg/kg) and xylazine (6 mg/kg) via the intraperitoneal (IP) route (22).

Acellularization of uterine tissue samples

Four different protocols (P1, P2, P3, and P4) were utilized for the decellularization of uterine tissues.

Protocols 1 and 2 (P1 and P2)

Rats were deeply anesthetised, uterine horns were excised, and connective tissues and fats were removed. Briefly, the horns were rinsed with phosphate-buffered saline (PBS, 25°C) incised in the mesometrium line and cut into rectangular pieces that varied in size depending on the test. Up to four samples from the same rat were immersed in 5 ml of SDS (1%) solution for 2 hours (P1) or 4 hours (P2) at room temperature. After SDS treatment, samples were washed for 1 week (P1) or 10 days (P2) using a washing buffer containing 0.05 M magnesium chloride hexahydrate, 0.2 mg/ml DNaseI (Roche, USA), 0.9% NaCl, and 1% penicillin and streptomycin on a shaker at 4°C (Table 1).

Protocol 3 (P3)

To prevent blood clots in the arteries during perfusion, rats were injected subcutaneously with heparin (100 IU/kg) one hour before anesthesia. Subsequently, the abdominal aorta was cannulated with a 20-G angiocatheter and cannulas were fixed with the 4-0 silk ligatures. The uterus was connected to 200 ml of heparin/PBS solution (50 IU/ml) and 200 ml PBS, respectively, and the volumetric flow rate was set at 4 ml/minutes. Then, their connection was cut off from the solution flow, and uterine horns were cut into rectangular samples. Uterine patches were soaked in three different doses of SDS (0.01%, 0.1%, and 1% in distilled water), respectively (the time of each dose was 24 hours). Samples were washed with PBS every 24 hours (3 times, 5 minutes, on a shaker) and finally soaked in 1% of Triton X100 for 30 minutes. In the end, the samples were washed with PBS (3 times, 10 minutes, on the shaker) (Table 1).

Protocol 4 (P4)

One hour before anesthesia, rats were injected subcutaneously with 100 IU/kg of heparin. Rats were anesthetised, and the diaphragm was dissected. Then ribs were cut, and a 50 ml syringe filled with NaCl was inserted in the left ventricle of the heart. The right atrium was cut to allow the flow. Subsequently, 200 ml of NaCl was slowly injected to remove the blood from the uterine tissue (until the lung tissue became white). Uterine horns were excised, connective tissue and fat were removed, and rinsed with PBS. The uterus was frozen at -20°C for 1 to 7 days. Next, the uterus was thawed, and one of the horns was attached to a peristaltic pump using a pipette tip and silicon tubing. Initially, perfusion with PBS was performed for one hour to remove cell debris. The perfusion speed was set at a flow rate of 10 ml/minutes. Then, five 24-hour cycles were performed. In the first two days, the cycle of SDS 1% (6 hours) and PBS (18 hours) was repeated. On the third day, the SDS time was reduced to 2 hours, the samples were treated with Triton X-100(1%) for 2 hours, and then PBS flowed for 20 hours, and this cycle was repeated until the fifth day (Table 1).

Table 1: Decellularization protocols

1.1: Decellularization protocol 1 and 2

Stages	Protocol 1	Protocol 2
SDS 1% (25°C)	2 hours	4 hours
Washing buffer	1 week	10 days

1.2: Decellularization protocol 3

Perfusion of heparin/PBS (50 IU/ml, 200 ml)

Perfusion of PBS (200 ml)

SDS 0.01%	24 hours
PBS	5 minutes (3 times)
SDS 0.1%	24 hours
PBS	5 minutes (3 times)
SDS 1%	24 hours
PBS	5 minutes (3 times)
Triton X100 1%	30 minutes
PBS	10 minutes (3 times)

1.3: Decellularization protocol 4

Perfusion of NaCl (200 ml) into the left ventricle

Physical treatment	Freeze/thawing of the uterus (at -20°C)		
Chemical treatment	Day 1	PBS	1 hour
		SDS 1%	6 hours
		PBS	18 hours
	Day 2	SDS 1%	6 hours
		PBS	18 hours
	Day 3	SDS 1%	2 hours
		PBS	10 minutes
		Triton X100 1%	2 hours
		PBS	20 hours
	Day 4	SDS 1%	2 hours
		PBS	10 minutes
		Triton X100 1%	2 hours
		PBS	20 hours
	Day 5	SDS 1%	2 hours
		PBS	10 minutes
		Triton X100 1%	2 hours
		PBS	20 hours

SDS; Sodium dodecyl sulfate and PBS; Phosphate-buffered saline.

Histological analysis

The presented cellular component and collagen fibers were evaluated by H&E and MT staining, respectively. To prevent any ECM breakdown, the segments of native and acellularized samples were fixed in 10% formalin for 24 hours. Subsequently, they were incubated in 70% ethanol at 4°C overnight and then dehydrated in graded alcohols. This was followed by immersion in xylene and embedding in paraffin wax. The paraffin-embedded tissues were cut into 5-μm slices and stained with H&E (23) and MT (24), using standard protocols.

DNA quantification

The efficacy of the acellularization process and the presence of nuclear materials were evaluated by measuring the remaining nuclear DNA. The native and acellular samples were dried using a freeze dryer (Zibrus vaco2, Germany) to remove the liquid and then, weighed less than 20 mg. The total DNA content was extracted using a commercial kit (DNeasy Blood & Tissue, Qiagen, Cat No. ID: 69504) as per the protocol provided. DNA concentration was calculated from OD measurements at 260 nm by picodrop (pico100, UK).

FESEM imaging

We performed FESEM to investigate the ultrastructure of the scaffolds in different protocols. To prepare the samples for FESEM imaging, we provided 0.5×5 cm^3 patches from the horns of native and acellular uteri of rats. The samples were first immersed in 2.5% glutaraldehyde solution at 4°C for 2 hours, washed with distilled water (DW) three times, and were dehydrated by graded series of ethanol (30, 50, 70, 90, 100%) each for 15 minutes and then dried with a freeze dryer (25). The samples were ultimately put under a scanning electron microscope (ZEISS Sigma 300 HV, Germany) following coating with the gold sputter-coater.

Cytotoxicity assay

Sample preparation

To ensure the complete removal of detergents from the scaffolds and to evaluate the cytotoxicity of the acellular scaffolds, equal-weighted round patches with 5 mm diameter were prepared. Patches were sterilized with 70% ethanol (24 hours) and washed in sterile PBS for the next 24 hours.

Cell culture

Rabbit mesenchymal stem cells (Ra-MSC) was purchased from the Iranian Biological Resource Center with accession cell No: IBRC C10723 which was confirmed as a rabbit by polymerasde chain reaction (PCR) method. Cryopreserved MSCs were thawed in a 37°C water bath. To improve cell viability, MSCs were put in a complete medium consisting of 10% fetal bovine serum (FBS, Gibco, USA) and 1% pen/strep. The cell-medium suspension was centrifuged at 2500 rpm for 10 minutes. The supernatant was recovered, and the pellet was resuspended in a 1 ml complete medium. After that, cells were counted by a hemocytometer and were seeded into a T25 or T75 flask at 5×10^4 cells/flask (T25) or 1×10^6 cells per T75 flask and incubated at 37°C and 5% CO_2. Every 3 days, the complete medium was changed. When the cells reached 80% confluency, they were ready for harvesting.

Direct MTT

Acellular scaffolds were put into a 96-well plate, and MSCs were seeded onto the scaffolds at the density of 2×10^4/w. The fresh culture medium containing Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) with 10% FBS and 1% penicillin/streptomycin was added to cell-seeded scaffolds. Cell viability was monitored 1st and 3rd day of culturing by MTT assay. In brief, after the prescribed period of incubation, the supernatant was removed, and the wells were washed twice with PBS. After that, 100 μL of MTT (0.5 mg/ml in PBS, Sigma Aldrich, USA) was added to each well and left on the plate for 4 hours at 37°C and a 5% CO_2 incubator. Then dimethyl sulfoxide (DMSO, DNAbiotech, Iran, 100 μL) was added to each well and allowed to react until the dissolution of the formazan pigment. The scaffolds were then removed, and their optical densities (ODs) were measured at 570 and 650 nm (as a reference wavelength) in a spectrophotometer.

The OD of the treated wells was compared with that of the control, which consisted of MSCs without any scaffold and the cell viability percentage was calculated according to the following formulas.

Equation (1): Viability=(OD sample–OD blank)/(OD control–OD blank)×100

Indirect MTT

MSCs were seeded onto wells of flat-bottomed 96-well plates at 10^4 cells/well. After 24 hours (to ensure the adherence of cells to wells), the acellularized scaffolds were added to the wells and incubated at 37°C for 24 or 72 hours. Thereafter, the scaffolds were removed from each well and the culture medium was discarded. MTT (100 μL, 0.5 mg/mL) was added to each well and the plates were incubated for 4 hours at 37°C. Then dimethyl sulfoxide was added, and the OD was measured like what we explained above (direct MTT).

Hemolysis assay

The effect of an acellularized uterine sample on red blood

cells (RBC) was determined by a hemolysis assay. Firstly, patches of uterine from all of the acellular protocols were cut (15 mg) and added to sample tube containing 4 ml NaCl 0.9% and preheated in a 37°C incubator for 30 minutes. Two tubes containing 4 ml NaCl 0.9%, and DW were considered as negative and positive control, respectively. Two ml of citrated blood were diluted with 2.5 mL NaCl 0.9%. Diluted blood (200 μl) was added to all sample and control tubes, and the tubes were replaced at 37°C for a further 60 minutes. The tubes were centrifuged at 1500 RPM for 10 minutes following the second incubation period. Release of hemoglobin which indicates the RBC damage was determined by photometric analysis of the supernatant at 545 nm (O.D). The percent hemolysis was calculated using the following formula.

% Hemolysis=(TS–NC)/(PC-NC)×100

TS: Absorbance of supernatant of erythrocyte suspension with the sample solution

NC: Absorbance of supernatant of erythrocyte suspension with NaCl

PC: Absorbance of supernatant of erythrocyte suspension with distilled water

This test was run in triplicate and the results were averaged.

Mechanical characterization

The mechanical properties or tensile test of the native tissue and acellularized scaffolds were conducted using a universal tensile machine (UTM) equipped with a 50-N load cell (Zwick/Roell, Model: Hct 25-400, Germany). Wet tissue samples (20 mm in length×5 mm in width) were pulled at a rate of 10 mm/minutes, displacement data were recorded, and a stress-strain curve was drawn. The tests were performed with three replicates for each sample (n=3). The ultimate tensile strength (UTS), Young's modulus (E), and Elongation at break (δ) were calculated using the following formula:

Equation (2): $E=(F/A)/(\Delta L/L0)$

Equation (3): $UTS=F/A$

Equation (4): $\%\delta =\Delta L/L0$

E=Young's modulus (Elastic Modulus)

δ=Elongation at break

F=Force exerted on an object under tension

A=Cross-sectional area

ΔL=Changes in length

L0=Initial length

Statistical analysis

The data were analyzed by SPSS software (version 23, IBM, USA) using the ANOVA test. Data were expressed as mean ± standard deviation, and a P<0.05 was considered as the significance level.

Ethical consideration

The experimental procedures were approved by the Animal Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC.1398.298).

Results

Histological evaluation

Investigation of acellularization efficiency in four different protocols by H&E staining demonstrated that only the P4 successfully removed cellular components in all epithelial, stromal, and smooth muscle layers. The samples processed by P1 and P2 showed the most residual cells compared to the others. In scaffolds obtained from P3, the nucleus of cells was removed but some cytoplasmic components remained. In the H&E staining the nuclei were purple and cytoplasmic components were pink, indicating the cytoplasmic components were not removed. Native uterine tissue was used as the control sample (Fig.1AI, II). Masson's trichrome staining showed that P1, P2, and P4 samples had an abundance of collagen fibers, similar to native tissue, that indicates no damage of detergents to the ECM; however, P3 specimens showed slightly reduced collagen fibers (Fig.1AIII).

DNA quantification

DNA quantification is an assay for assessing the efficiency of cell removal protocol. DNA content of samples was reported as a percentage of normal (intact) tissue. The remaining DNA contents of all the protocols were significantly lower than native tissues, while P1 samples showed the highest amount of DNA remnants after the decellularization process compared to other protocols (P<0.05, Fig.1B).

FESEM results

As observed in the low magnification of FESEM images, the scaffolds, after acellularization by P1, P2, and P4 protocols, appeared as porous sponges with a well-defined three-dimensional structure. Even the structure of the vascular conduits in protocol 4 was preserved with intact conformation. Although scaffolds prepared using P1 and P2 protocol looked rich in fibrous content, P4 appeared more porous than other decellularization scaffolds. P3 became collapsed in all layers and showed poor porous structure (Fig.2A, B). Cross-sections of the native uterus and acellular scaffolds from each protocol viewed at high

magnification showed tissue fibrils (e. g. collagen and elastin) that maintained their striated and bundle patterns (Fig.2C).
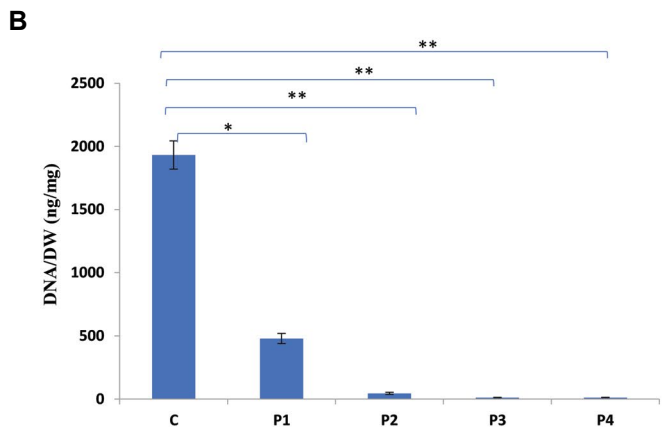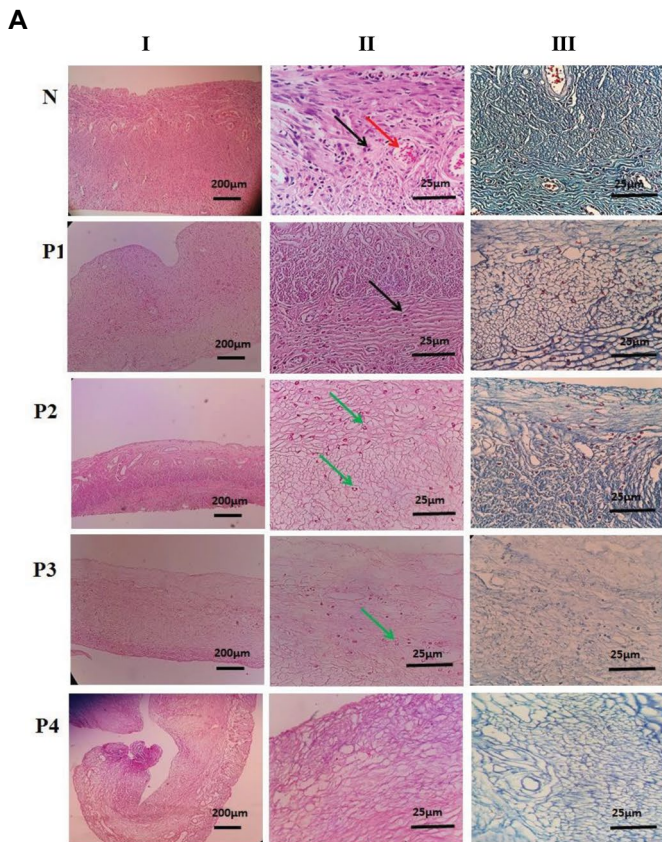

Fig.1: Verification of decellularization by histological staining and residual DNA content. **A.** Histological assessment of decellularized uterine scaffolds (with different protocols; P1, P2, P3, P4) and native tissue (N). Optical microscope images of hematoxylin-eosin staining indicate cells (I and II) (black arrowhead) in the layers of native (N) samples and blood cells in their arteries (red arrowhead). In P1 and P2 protocols, the cells were partially removed; however, some cytoplasm without a nucleus remained (green arrowhead). Also, in protocol 3, the cytoplasm without a nucleus is seen. Only in protocol 4, the cells are completely removed. III; Masson's trichrome staining demonstrated that there is no damage in the collagen fibers of the scaffold from P1, P2, and P4 following the decellularization process but in scaffolds obtained from P3, fibers are decreased (I; 10x magnification, II and III; 40x magnification). **B.** Quantification of residual DNA showing a drop of 24, 2.3, 0.5, and 0.6% for the protocols 1-4 respectively compared to native sample (100%). *; P=1.1×10^{-10} , **; P=2×10^{-12}, and DW; Dry weight.
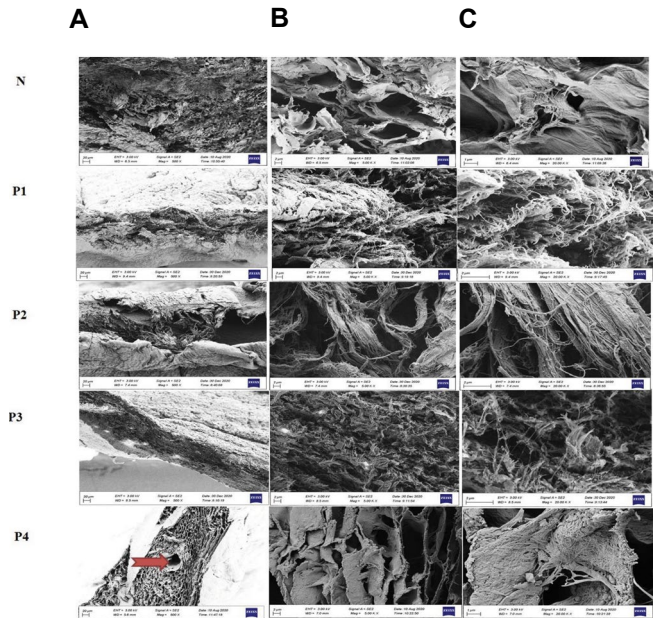

Fig.2: Scanning electron microscope images of the cross-section of native (N) and acellularized protocols (P1-P4) of rat uterine tissues. **A, B.** Scanning electron micrographs at lower magnification (200 and 5000x magnification) showing pores of scaffolds. **C.** Higher magnification (20000x magnification) demonstrates the fibers bundles. Arrowhead indicates blood vessel.

MTT assay results

Because incomplete removal of detergents from a scaffold can affect cell viability *in vitro* and *in vivo*, we evaluated the biocompatibility of scaffold by MTT assay. In the direct method, the cell viability of MSCs that were seeded on all the P1, P2, P3, and P4 acellular scaffolds was significantly higher than in the scaffold-free control group (Fig.3A). In indirect contact of the scaffold with MSCs, no significant decrease was seen in cell viability compared with the control samples at 24 and 72 hours (Fig.3B).

Hemocompatibility

Results obtained for hemolysis of citrated blood with acellularized uterine patches are shown in Table 2. The percentage of hemolysis in the presence of uterine scaffolds prepared from all of the protocols was less than 2%. According to the Autian report (26), when the material presents a hemolysis percentage below 5%, it is considered a non-hemolytic material. Therefore, it can be concluded that acellularized uterine from all 4 protocols could be considered highly hemocompatible.

Table 2: Hemolysis of blood by acellularized uterine patches prepared from 4 protocols (P1, P2, P3, P4)

Sample	Optical density at 545 nm	Hemolysis (%)
Water (control+)	1.31	100
Saline (control-)	0.055	0
P1	0.06	0.39
P2	0.07	1.19
P3	0.058	0.23
P4	0.055	0

Mechanical characterization of scaffolds

A mechanical evaluation test was conducted to ensure that the acellular process did not affect the structural integrity of scaffolds. The extracted results from the stress-strain curves (Fig.4A) are shown in Figure 4B-D.

The native uterus has the maximum tensile stress of 0.66 ± 0.1 MPa and Young's modulus of 1.7 ± 0.2 MPa and they are higher than acellularized samples (P1, P2, P3, and P4), but these differences are not significant (P=0.2). Also, elongation at break was only significantly different for P3-treated uterine tissue which led to a decreased tissue extensibility (P=0.04).
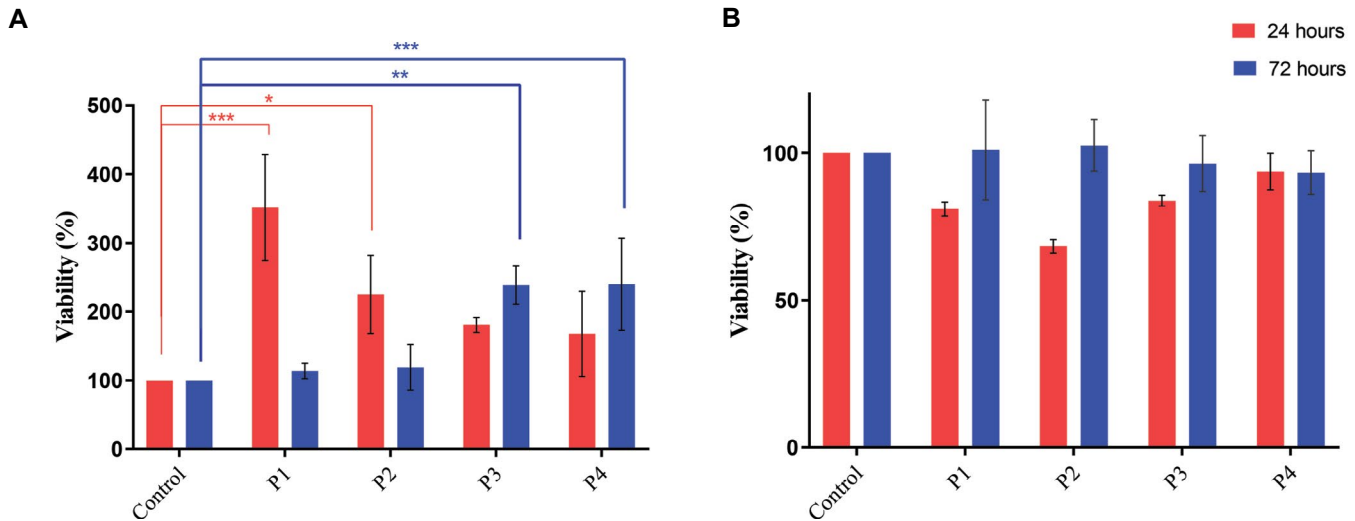

Fig.3: Analysis of cell viability following culture on acellularized uterine scaffolds. **A.** The MTT photograph in direct contact revealed that there were higher cell viabilities in the acellularized samples (P1- P4) compared with control groups. **B.** In indirect contact there were no significant differences between the acellularized groups (P1- P4) and control group after 24 and 72 hours. *; P=0.002, **; P=0.0002, and ***; P=0.0003.
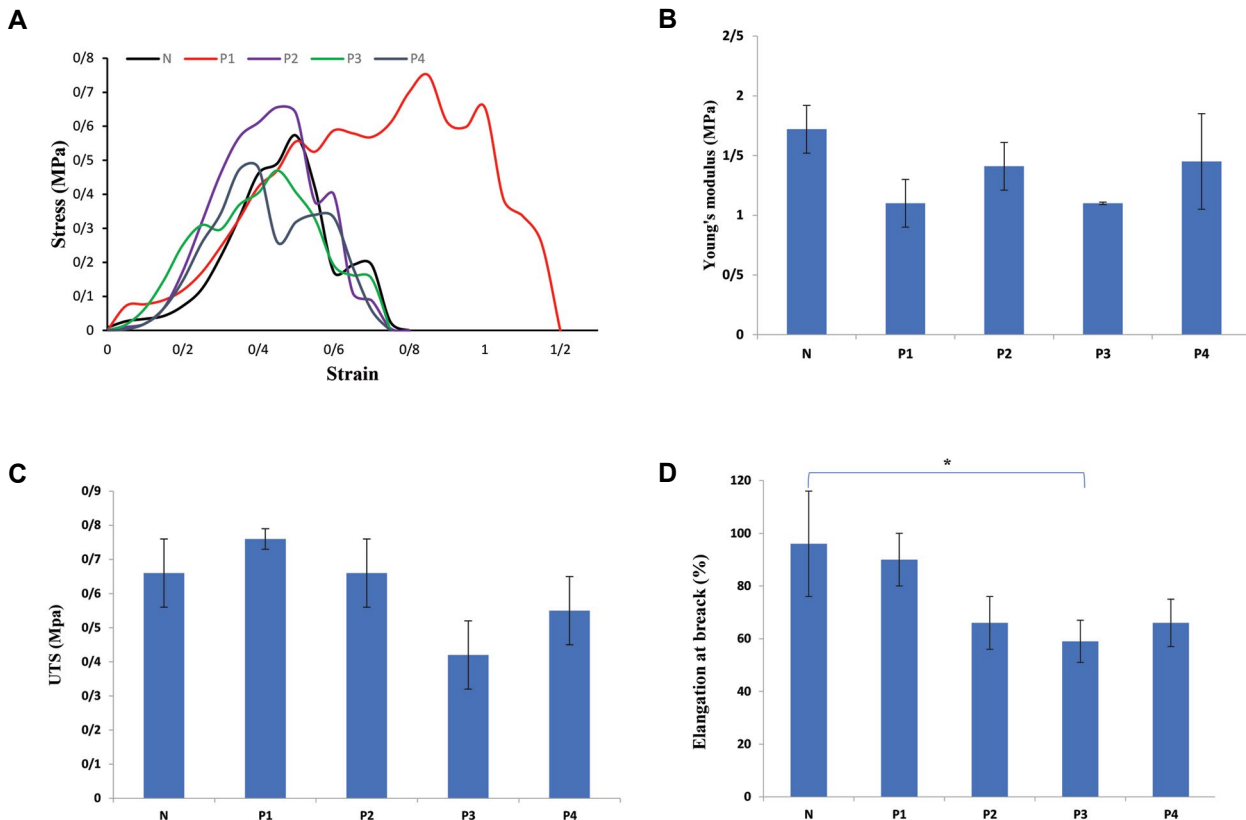

Fig.4: Mechanical evaluation of the samples (n=3); native (N) and acellularized tissues prepared from protocols 1-4 (P1, P2, P3, and P4). **A.** Stress-strain curves, **B.** Young's modulus, and **C.** ultimate tensile strength of acellularized specimens are not significantly different from each other, or the native group. 1 MPa (Mega Pascal)=1 N/mm². **D.** Elongation at break in P3 was significantly different compared to native (*; P=0.04).

Discussion

Tissue engineering has proposed various biomaterials for repairing different tissues. Each of these biomaterials has advantages and disadvantages. Among these biomaterials, decellularized ECM-based constructs are more successful due to their high similarity to natural tissues (biocompatibility, mechanical properties, etc.). Moreover, as they have properties such as low antigenic properties and lack stimulation of immune responses in tissue regeneration, can demonstrate additional useful applications (27).

Since the use of natural decellularized scaffolds is currently a promising potential for regenerating, replacing, or improving the function of various organs such as the vagina, heart, and bladder, researchers are working to achieve an optimal protocol of tissue engineering for uterine tissue repair and treatment of AUFI patients. Previous studies compared the efficacy of different protocols such as perfusion of detergents and ionic solutions or high hydrostatic pressure for decellularization of the different animal uterine tissues (15, 16, 28). Optimizing the cell removal protocol is very important because tissue standardization will enable the optimization of clinical protocols, which will result in better outcomes for patients. To achieve the protocol that while is simple, has similar characteristics to the natural tissue, we selected four different types of decellularization methods and evaluated the efficiency of cell removal and the preservation of the physicochemical properties as well as the biological integrity of rat uterine tissues. In protocol 1, we used SDS 1% for 2 hours, and DNase to remove the cells and nuclei. In protocol 2, the soaking time of scaffolds in the SDS was increased to 4 hours. In protocol 3, we used three additional doses of SDS (0.01, 0.1, and 1%) with a longer time (24 hour) and Triton X100. In protocol 4, in addition to cardiac perfusion and removal of blood cells, we performed a freeze-thawing process before using the detergent (SDS and Triton x100). Because the success of the decellularization method is based on the cell removal while maintaining ECM, we evaluated these two properties.

Histological examination showed that protocol 1 still had some cell residue and even the amount of DNA was 24% compared to the native sample. In protocol 2, with increasing detergent time from 2 to 4 hours and washing duration (from 7 to 10 days), the number of complete cell debris (nucleus and cytoplasm) decreased, and the amount of DNA decreased considerably, but their cell cytoplasm remained. The importance of complete removal of cellular residue from scaffolds is related to the presence of major histocompatibility complexes (MHC) present on the cell membrane, which can lead to immune responses and hyperacute rejection of scaffolds (29). These nucleus-free cytoplasms also seemed to be removed if the washing period was longer. The DNA count of scaffolds obtained from protocol 3 was the lowest but the cytoplasm remained to some extent. Interestingly, although DNase enzyme (DNA digester) was used only in the first two protocols,

the scaffolds obtained from protocol P3 had the lowest amount of DNA, which shows that the using of detergents (SDS and Triton X-100) is enough to remove DNA. Our study showed that protocol 4 was the most efficient method of cellular removal (without any debris).

The second purpose of effective decellularization is preserving biologically active ECM components. Successful repair after implantation of acellular tissue depends on the preservation of the 3D ECM structure or its protein composition. In the regeneration process, ECM has an essential role in cell adhesion, aggregation, migration, as well as proliferation and differentiation of cells for that specific tissue. This is done by preserving vascular cells and molecules such as growth factors, collagen, elastin, glycosaminoglycans, fibronectin, and, laminin (30). Since collagen is one of the main components of ECM proteins that plays an important role in providing the tissue framework connection, and tensile strength and, affecting cell types disposition, the destruction or reduction of this protein is the cause of scaffold failure (31). Histology and FESEM analysis of protocols 1,2 , and 4 showed that ECM proteins preserved their structural integrity during decellularization, but similar to Santoso et al. (16) study, long exposure time with SDS, had a disruptive effect on the ECM during the process of decellularization and reduced the collagen bundles in P3. Another important role of the acellular ECM in the repair process is mechanical support for tissue regeneration and fabrication and even strategies such as the use of cross-linkers were used to optimize the mechanical properties and structural stability of the tissue (32). Scaffold stiffness plays an important role in the migration and proliferation of stem cells and modulation of the ECM microenvironment after recellularization. Tiemann et al. (33) suggested that the sheep uteri tissues became stronger after decellularisation treatments with SDS, SDC, or triton x100. They explained that this is the result of the denser structure of the ECM caused by cell removal.

In our study, although uterine tissue appeared to be visually thinner and weaker after the acellularization, there were no statistically significant differences between these acellularized samples from all of the protocol and native groups in young modulus elasticity and UTS. However, the elongation at break of the samples obtained from the p 3 protocol is significantly lower compared to the normal sample. Reduction and destruction of collagen (in histological examination) and contraction of ECM fibers (in FESEM photos) justify this extension reduction.

Since increased detergent concentrations in the scaffold in addition to ECM disruption, can increase adverse cell outcomes (23), we assessed the removal of the detergent agents and toxicity of the scaffolds by MTT assay (via direct and indirect contacts of MSCs to acellularized scaffolds) and hemolysis assay. The results of the MTT assay showed that 24 h after incubation, the acellular scaffolds from all protocols did not inhibit the growth of the co-cultured cells but rather promoted cell proliferation

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significantly. The three-dimensional environment of the scaffolds (compared to the two-dimensional environment of the control group), can be a justification for this finding. Yu et al. (34). showed that growth factors such as epidermal growth factor (EGF), transforming growth factor-beta (TGF-β) and basic fibroblast growth factor (bFGF) were preserved after pancreatic body tail acellularization and these cytokines can stimulate proliferation of cells in MTT assay. A previous study showed that SDS 1% altered the basement membrane complex (BMC) of the porcine urinary bladder. BMC has an important function in the attachment and growth of cells (35). In our study, Scaffolds obtained from protocols 1 and 2 were treated with SDS for a shorter time (than the other two protocols). Therefore, the possibility of more cell proliferation in the first 24 hours can be attributed to the more intact structure of ECM and better cell attachment in these two groups. Increased proliferation in 24 hours, caused a lack of nutrients, growth factors, and insufficient space, followed by cell death in 72 hours.

Also, in the indirect MTT test, where the cells were not implanted in the three-dimensional structure of the scaffolds, cell proliferation was not significantly different from the control sample. These results of the MTT assay suggested that the acellular uterine scaffolds exhibited good compatibility without any cytotoxic effect on co-cultured cells. Another test for the assessment of the cytotoxicity of ECM is the hemolysis assay. If toxic chemical agents are not removed from the scaffold during acellularization, it will cause RBC damage and hemoglobin release (36). So, a low degree of hemolysis indicates better hemocompatibility of the biomaterial. In our study, uterine patches from all protocols were hemocompatible. SDS is the strong ionic detergent and preferred agent for removing cell debris from different tissue. However, in addition to the damage to phospholipid membranes (37) and denaturation of proteins (38), SDS can lead to cell toxicity (39).

Momtahan et al. (36) removed SDS using DW, Phosphate-buffered saline, and Triton X-100 from decellularized heart tissues. They showed that using Triton-X 100 can double the SDS removal rate from ECM (36). Nouri et al. (40) used three methods for the decellularization of mouse uterine. 1: SDS and triton X-100 solutions (at concentrations of 0.1, 0.3, 0.6%, each for 24 hours), 2: hypertonic and hypotonic solutions, and 3: freeze and thaw and subsequently in 0.5% SDS and Triton X-100 0.1% (each for 48 hours). Their data indicated SDS and Triton X-100 (unlike the other two methods) could completely decellularize the uterus. 3D ultrastructure of decellularized scaffold prepared by this protocol remained intact and the MTT assay revealed that these uterus scaffolds were non-toxic for cell growth and proliferation of menstrual blood stem cells.

In summary, according to the results of our study, P1 and 2 due to incomplete cell removal and P3 due to ECM damage do not have the necessary criteria to be introduced as an optimized method of acellularization of uterine tissue. Therefore, we suggest that the P4 process is an optimal protocol for the acellularization of rat uterus (out of 4 protocols introduced) with complete removal of cell debris, preservation of the protein fibers of ECM, removal of cytotoxic detergents, and finally, preservation of mechanical characteristics.

Conclusion

We aimed to evaluate and ultimately propose an efficient decellularization protocol for uterine tissue, but this study is required more evaluations of recellularization and biocompatibility of the scaffold. Also, not reporting the level of proteins (such as elastin, and fibronectin) and not evaluating the vascular network were the limitations of this study. Evaluation tests should also be performed after implantation *in vivo* studies.

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Authors' Contributions

M.M.K.; Conceptualization, data curation, formal analysis, validation, experimental work, and writing-original draft. M.S.; Conceptualization, review,editing, formal analysis, and validation. F.N., S.R.; Conceptualization, formal analysis, review and editing. S.R., M.S.; Data curation, validation, and project administration. All authors read and approved the final manuscript.

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