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Preparation and characterization of human decellularized ovarian scaffold based on supercritical carbon dioxide protocol

Fatemeh Hosseinpour[1], Ali Zeinolabedini Hezave[2,3], Tahereh Talaei-Khozani[1,4], Mojtaba Rastgou-Maeini[1] and Ashraf Hassanpour-Dehnavi[1,4,5*]

*Correspondence:
a_hassanpour@sums.ac.ir

[1] Tissue Engineering Lab., Anatomy Department, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
[2] Fanavari Atiyeh Pouyandegan Exir Company (APEX Technologies Co.), Science and Technology Park, Arak, Iran
[3] Fanavari Arena Exir Sabz Company (AREX Green Technologies Co.), Arak, Iran
[4] Histomorphometry and Stereology Research Center, Anatomy Department, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
[5] Department of Anatomical Sciences, School of Medicine, Imam Hussain Square, Zand St., Shiraz 7134845794, Fars, Iran

Abstract

Background: Primary ovarian insufficiency affecting 1–3% of women under 40, causes premature menopause and estrogen deficiency. With increasing life expectancy, a large percentage of women also face estrogen-related symptoms. A bioengineered ovary is one of the strategies to replace or enhance ovarian tissue function. A key advancement in bioengineering is the development of ovarian decellularized extracellular matrix scaffolds that mimic natural ovarian niche. Recent studies indicate that supercritical carbon dioxide ($scCo_2$), with a density comparable to liquids and viscosity and diffusion coefficients properties similar to gases, holds substantial promise for application in engineered scaffolds. Therefore, we established a human decellularized ovarian scaffold based on a $scCo_2$ process, as an optimized protocol.

Methods: We evaluated two distinct pressure conditions (200 and 300 bar), while maintaining identical thermal (40 °C) and temporal (1.5 h) parameters, during the $scCO_2$ decellularization process. In addition, two modifications were implemented to identify the most optimal protocol for enhancing the decellularization process: the inclusion of 70% ethanol as a co-solvent and the application of 1% sodium dodecyl sulfate (SDS) as a pretreatment for 4 h while utilizing the $scCO_2$ system under the previously established conditions. Cell removal was confirmed by DNA quantification and H&E staining. Extracellular matrix structure evaluated by histological staining and scanning electron microscopy (SEM). Glycosaminoglycans (GAGs) content was quantified using a dimethyl methylene blue assay following extraction with HCL and MTT test was conducted to evaluate scaffold's cytocompatibility.

Results: Application of 1% SDS, while utilizing the $scCO_2$ system at 200 bar and 40 °C for 1.5 h, established an optimal protocol for preserving the essential characteristics of the ovarian ECM. This protocol is able to meet previously established decellularization criteria and histological staining and SEM showed that the ECM architecture was satisfactorily preserved. GAGs quantification indicated adequate preservation of GAGs content. Finally, MTT test presented the scaffolds had suitable cytocompatibility.

Conclusions: We propose an optimal protocol utilizing 1% SDS as a pretreatment, followed by the $scCO_2$ system. This protocol addresses common challenges

associated with traditional decellularization methods and presents a promising avenue for advancing ovarian tissue engineering applications.

Keywords: $scCO_2$, Ovary, Decellularization, ECM-based scaffolds, Tissue engineering

Introduction

Women today are experiencing longer life expectancies [1], which often means spending a significant portion of their lives in the post-menopausal stage, experiencing short-term (hot flashes, insomnia, etc.) and long-term (infertility, osteoporosis, cardiovascular diseases, etc.) [2] symptoms of decreased estrogen. In this context, women with premature menopause and primary ovarian insufficiency (POI), who undergo menopause before the age of 40 [3] (1–3% of women), experience these conditions much earlier [4–7]. Historically, more than 70% of cases of POI, also referred to as premature ovarian failure (POF), were considered idiopathic [8]. However, chromosomal abnormalities, such as Turner syndrome or Fragile X syndrome, autoimmune disorders (conditions, such as autoimmune thyroid disease or Addison's disease) and metabolic (galactosemia) disorders, ovarian surgery, chemotherapy, or radiation therapy, infections such as mumps, mitochondrial and oxidative stress, have been known to cause it [9, 10].

Recent advancements in treatment approaches for POI and premature menopause include Hormone Replacement Therapy (HRT) [11, 12], ovarian tissue cryopreservation [13], in vitro activation (IVA) [14] and stem cell therapy [15]. However, it is important to note that these interventions do not fully restore normal ovarian function or significantly enhance reproductive success [16]. Long-term application of HRT is fraught with risks of various cancers and cardiovascular diseases, for which it is unsuitable for many women. Techniques such as IVA and cryopreservation aim to stimulate dormant primordial follicles to promote natural conception; however, concerns about follicle depletion and egg quality remain [17–20].

The construction of bioengineered ovaries represents another promising strategy that researchers have been actively working on recently. In general, the aim of this strategy is to fabricate biological substitutes to replace or improve ovarian tissue function, and it is scaffold-based, growth factor-based, and stem cell-based. Among these, the natural scaffold is considered as an important factor, since it provides a specialized microenvironment and mechanical framework that may support cell proliferation and differentiation [21–23].

A notable advancement in this area is the development of ovarian decellularized extracellular matrix-based scaffolds, which potentially can mimic the natural ovary aspects, such as induction of the steroidogenesis and folliculogenesis [6, 24].

Among the decellularized methods, supercritical carbon dioxide ($scCO_2$) has emerged as a promising decellularization method due to its efficiency in removing cellular material while maintaining the integrity of the extracellular matrix, as demonstrated in cardiac, vascular, and hepatic tissues [21, 25, 26].

$scCO_2$ possesses unique properties (low critical pressure of 7.38 MPa and temperature of 31.1 °C) allowing this gas to penetrate even very dense materials and to become a strong solvent while being chemically inert, non-toxic, and easily removable without leaving residues. Moreover, this can reduce decellularization time, enhance the diffusion rate, and sterilize biomaterials without alteration of its composition [26, 27].

$scCO_2$ was reported to destroy cells within tissues (e.g., porcine heart valves and cartilage), at pressures of 200–300 bar and temperatures of 35–40 °C, while many critical extracellular matrix (ECM) proteins remained preserved, which may enable this process to be at least as effective or even superior as compared with all existing decellularization methods [28, 29]. However, the efficiency of $scCO_2$-assisted decellularization is influenced by the tissue's cellularity, density, lipid content and operational parameters, such as pressure and temperature; minor pressure changes can drastically alter density and solubility, while increasing pressure positively impacts efficacy until a plateau is reached [25, 30–33]. Hence, this study focused on developing a novel $scCO_2$-based protocol for optimizing the decellularization of human ovaries. Results arising from this approach could provide some hope for fabricating an artificial ovary for restoring human fertility and ovarian function.

Results

Decellularized process assessments

Phase one of experiment

In the initial phase of the experiment, samples were subjected to the $scCO_2$ system under two distinct pressure conditions (200 and 300 bar) while maintaining identical thermal (40 °C) and temporal (1.5 h) parameters. Under both pressure conditions, the samples retained their shape and homogeneity without any deformation, exhibiting a pale pink coloration that indicated the early stages of cellular component removal. The DNA quantification assay demonstrated a significant reduction in DNA content, with an approximate decrease of over 80% compared to the native samples, which had a DNA content of 2038.036 ng/mg dry tissue weight (Fig. 1). However, despite this considerable reduction, the protocols employed did not fulfill the previously established decellularization criteria, such as the absence of nuclear material in the tissue sections (Fig. 3B) and a residual DNA content of less than 50 ng dsDNA/mg dry ECM weight [34].

Phase two of experiment

In the second phase of the experiment, two modifications were implemented to identify the most optimal protocol for enhancing the decellularization process. The modifications involved one group that utilized the addition of 70% ethanol as a co-solvent, and another group that applied 1% sodium dodecyl sulfate (SDS) as a pretreatment, all while employing the supercritical $scCO_2$ system under previously established conditions.

After the decellularization process by optimal protocol (1% SDS pretreatment + $scCO_2$ at 200 bar and 40 °C for 1.5 h), a color transition of ovarian tissues from red to white and transparent appearance was seen (Fig. 2A, B).

DNA content of samples treated with $scCO_2$ combined with ethanol, as well as those subjected to $scCO_2$ at 200 bar, exceeded the threshold for successful decellularization (< 50 ng/mg dry weight). In addition, no significant difference in DNA content was observed between these groups. In contrast, a substantial reduction in DNA content (30.33 ng/mg dry weight) was achieved following the application of 1% SDS for 4 h as a pretreatment in conjunction with the $scCO_2$ system, thereby successfully meeting our established standard for effective decellularization (Fig. 2C).

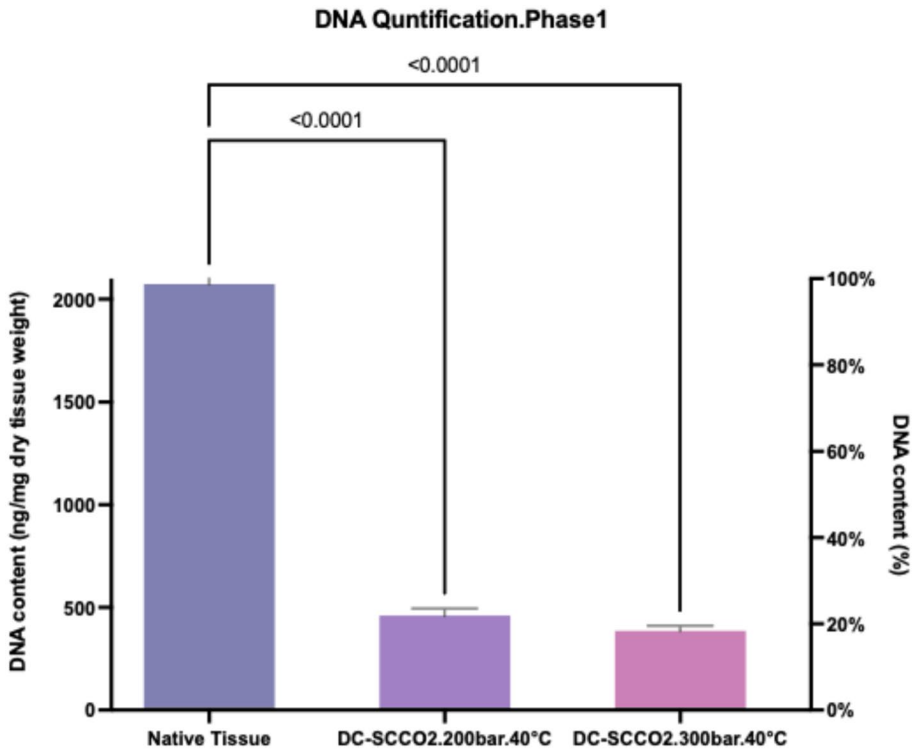

Fig. 1 DNA quantification assay demonstrated a significant reduction in DNA content in the scCO2 system under two distinct pressure conditions (200 and 300 bar), while maintaining identical thermal (40 °C) and temporal (1.5 h) parameters compared to the native tissues. (Data are expressed as the mean ± standard error of the mean (SEM), $n = 3$ per group)

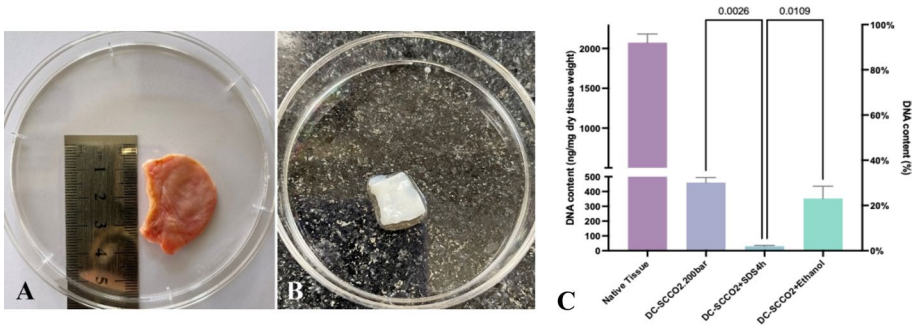

Fig. 2 Color transition of ovarian tissues after decellularization by optimal protocol (1% SDS pretreatment + scCO2 at 200 bar and 40 °C for 1.5 h); **A** Native ovarian tissue cortex, **B** Decellularized scaffold, **C** DNA quantification assay demonstrated a significant decrease in the DNA content after using 1% SDS for 4 h as a pretreatment with scCO2 system at 200 bar and 40 °C for 1.5 h. (Data are expressed as the mean ± standard error of the mean (SEM), $n = 3$ per group)

Characterization of the ECM preservation

Hematoxylin and eosin (H&E) staining

A remarkable devoid of nucleic materials was noted as the developed decellularization methods proceeded. The presence of multiple cavities within the tissue slices in the $scCO_2$ + SDS4h group serves as a reliable indicator of successful oocyte and stromal cells extraction. Furthermore, the preservation of overall tissue architecture and

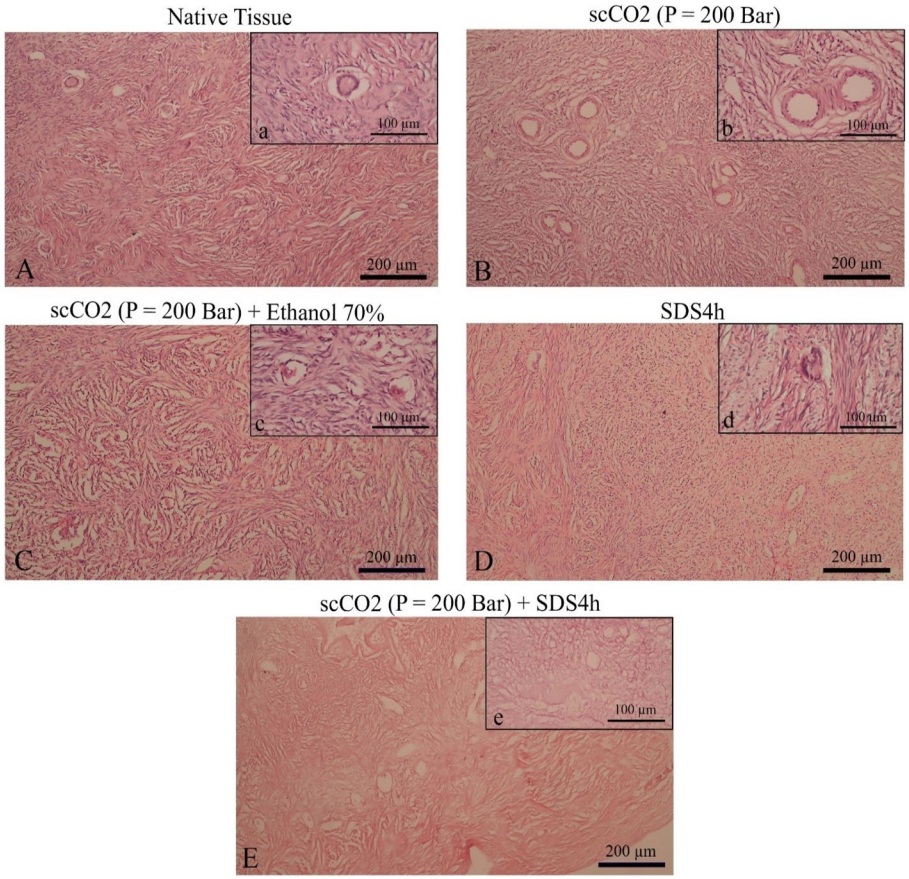

Fig. 3 Hematoxylin and eosin staining revealed a lack of nucleic materials and preservation of tissue architecture after a 4-h pretreatment with 1% sodium dodecyl sulfate (SDS) followed by treatment in the supercritical carbon dioxide (scCO2) system at 200 bar and 40 °C for 1.5 h (**E**), in contrast to the native tissue (**A**). In comparison, the presence of numerous nuclei and intact cellular structures in tissues decellularized using other methods indicates the inadequacy of these protocols (**B**–**D**). *P* Pressure

integrity is deemed adequate (Fig. 3E). The H&E staining clearly showed that the other decellularization methods were insufficient to achieve effective decellularization, as evidenced by the presence of numerous nuclei and intact cellular structures within the tissue (Fig. 3B–D).

Gomori's aldehyde fuchsin staining

Gomori's aldehyde fuchsin staining revealed the presence of tense purple elastic fibers, which appeared more pronounced around blood vessels. As illustrated in Fig. 4B, the staining demonstrated the persistence of elastic fibers that scattered throughout the decellularized tissue (1% SDS pretreatment + $scCO_2$ at 200 bar and 40 °C) with the same architecture, comparable to native tissue.

Masson's trichrome staining

The Masson's trichrome staining revealed a significant presence of collagen as the most abundant protein in the ovarian interstitial matrix, in both native and decellularized tissues. The fibers were distributed throughout the decellularized cortex and medullary

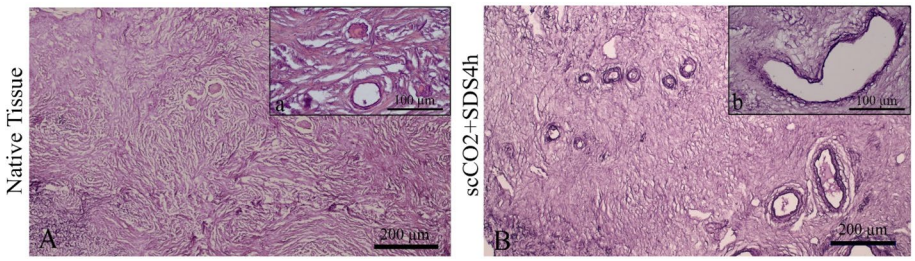

Fig. 4 Gomori's Aldehyde Fuchsin staining in scCO2 + SDS4h group indicated a noticeable amount of elastic fibers (tense purple color) preserved within the tissue apparently more pronounced around blood vessels (**B**), comparable to native tissue (**A**)

regions, with elevated concentrations observed in the tunica albuginea.The distribution and structural integrity of collagen fibers in the decellularized ovary were found to be comparable to those observed in the native tissue (Fig. 5).

Periodic acid–Schiff (PAS) staining

PAS is a special staining procedure used to locate carbohydrates, especially glycoproteins and polysaccharides in tissue sections by an intensified magenta color. The strong staining of native tissue slides was indicative of richness of carbohydrate-containing structures in the ovarian tissue, while it was relatively preserved in decellularized tissue (Fig. 6).

Alcian blue staining

Alcian Blue staining imparts a turquoise color to GAGs and specifically highlights acidic mucopolysaccharides, which are important components of the extracellular matrix. It

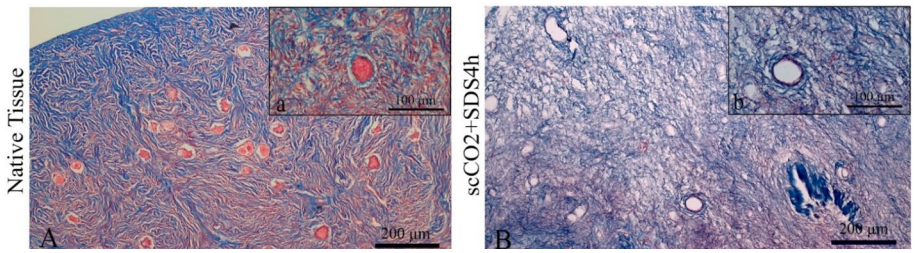

Fig. 5 Masson's Trichrome staining revealed well preservation of structural integrity of collagen fibers (Navy blue color) in scCO2 + SDS4h decellularized group (**B**). Collagen fibers were distributed throughout the decellularized cortex and medullary region, comparable to native tissue (**A**)

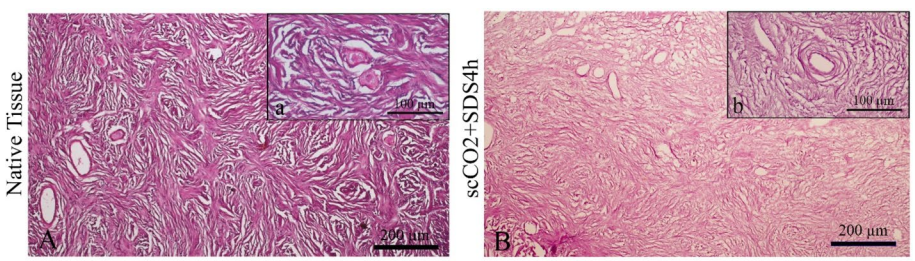

Fig. 6 PAS staining reveals relatively preservation of carbohydrate-containing structures indicated by magenta color in scCO2 + SDS4h decellularized group (**B**) compared to the native tissue (**A**)

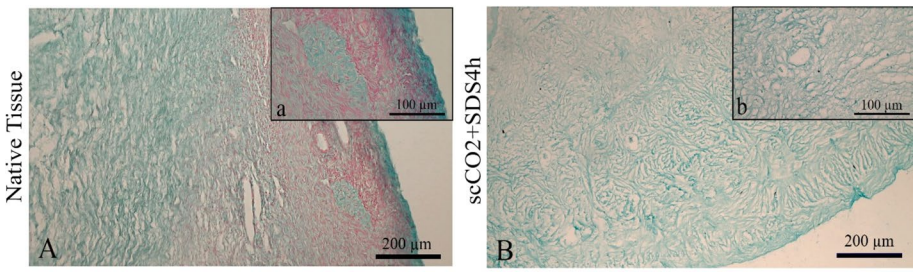

Fig. 7 Alcian Blue staining revealed that the content of glycosaminoglycans, indicated by the turquoise color, was effectively preserved, with a higher concentration observed in the ovarian cortex of the scCO2 + SDS4h decellularized group (**B**), comparable to native tissue (**A**)

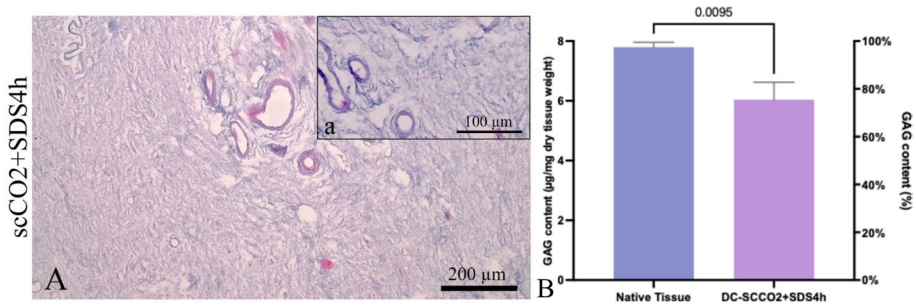

Fig. 8 **A** Alcian Blue and Periodic Acid-Schiff staining is employed to assess the preservation of glycosaminoglycans (GAGs) (blue-violet) and carbohydrates (magenta) within decellularized ovarian tissues by scCO2 + SDS4h system. **B** GAGs quantification shows reduction of GAGs compared to the native tissue; however, more than 70% of the GAGs content was successfully preserved

revealed that the GAGs content was effectively preserved, with higher color intensity observed in the ovarian cortex of decellularized tissue (Fig. 7). Furthermore, the combination of Alcian Blue and PAS staining exhibited a marked preservation of GAGs and carbohydrates within decellularized ovarian tissues (Fig. 8A).

GAGs quantification

Glycosaminoglycans content was also quantified in this study. The reduction of GAGs compared to the native tissue was significant ($p = 0.0095$); however, more than 70% of the GAGs content was successfully preserved (Fig. 8B).

Scanning electron microscopy (SEM) assessment

In addition to the findings from light microscopy, SEM assessments demonstrated the preservation of microarchitectural integrity and effective removal of cells following decellularization. Lower magnification images of the decellularized ovarian scaffolds revealed ovarian surface epithelium and a complex network of fibers interspersed with porous structures that were previously inhabited by various cell types. At higher magnification, the ECM framework appeared largely intact in several regions, including the tunica albuginea and areas formerly occupied by primordial and growing follicles, as well as blood vessels (Fig. 9).

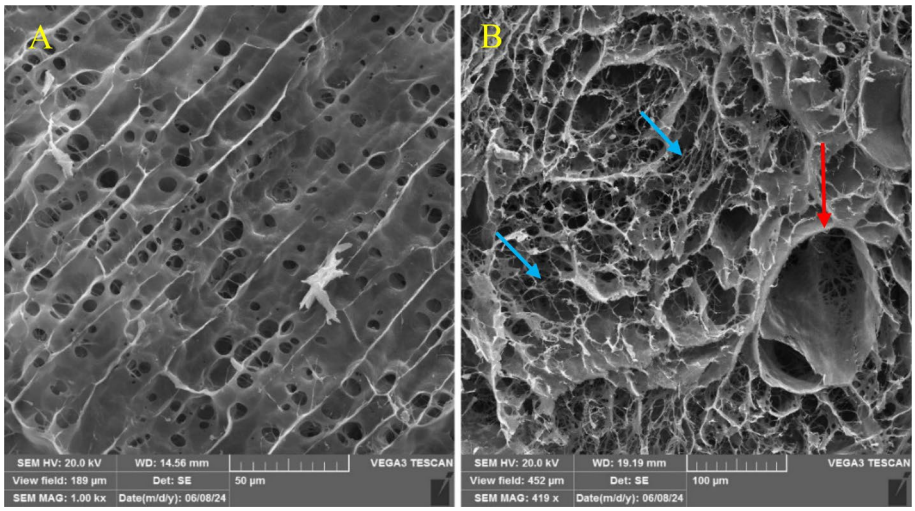

Fig. 9 Scanning electron microphotographs demonstrate ovarian surface epithelium (**A**), efficient devoid of cells with good preservation of 3D structures and the collagen fibers (blue arrows) orientation as well as blood vessels (red arrow) (**B**)

Cytotoxicity assay

MTT assay revealed that the decellularized scaffold was non-toxic and human Wharton's jelly mesenchymal stem cells (HWJMSCs) seeded on the scaffolds remained viable throughout the culture period. Comparison of optical density (OD) values of eluted formazan over several days indicated higher metabolic activity of cells seeded onto the scaffold. On the first day of culture, metabolic activity of cells in the decellularized scaffold was comparable to that observed in conventional 2D culture. However, at subsequent intervals, the OD values recorded from the scaffolds were significantly higher, suggesting that HWJMSCs exhibited an enhanced metabolic activity rate and normal proliferation behaviors within the decellularized scaffold (Fig. 10).

Discussion

Engineered scaffolds represent one of the three fundamental components in tissue engineering research, providing an optimal environment for cell adhesion, migration, and proliferation. The successful implantation of cells within tissue engineering frameworks necessitates that scaffold materials exhibit at least four essential characteristics: (1) excellent cell compatibility; (2) defined mechanical strength and deformability; (3) a three-dimensional porous architecture; and (4) ease of sterilization and storage [36].

A significant advancement in the fabrication of bioengineered ovaries is the emergence of scaffolds derived from decellularized ECMs, which have the potential to replicate key features of natural ovarian tissue [6]. The decellularization process involves the removal of cellular components, including lipid membranes and genetic material while retaining the structural integrity of the ECM [37]. The effectiveness of this process hinges on achieving a delicate balance between the complete elimination of cellular constituents and the preservation of ECM components, which are

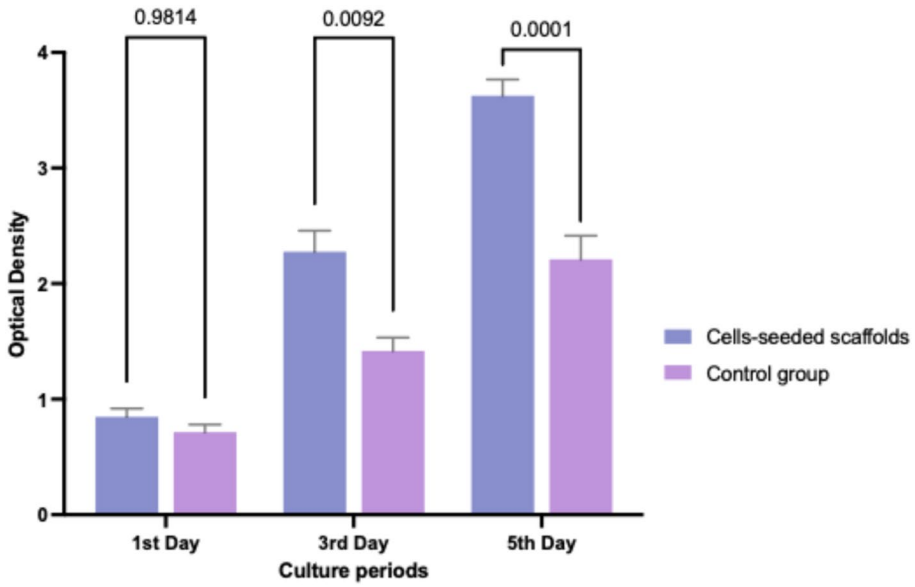

Fig. 10 MTT assay demonstrates cytocompatibility of cells-seeded scaffolds. On the first day, metabolic activity of cells seeded onto the scaffold was comparable to the control group, whereas, at longer culture intervals, a significant higher optical density value was detected. Data are expressed as the mean ± standard error of the mean (SEM)

crucial for maintaining the three-dimensional architecture necessary for biomimetic tissues or for use as foundational materials in more sophisticated biomaterials [38]. Various decellularization techniques have been developed for ovarian tissue, ranging from straightforward physical methods to more complex chemical and enzymatic approaches. A primary challenge associated with these materials is the establishment of an appropriate protocol that effectively removes cellular components while adequately preserving ECM functionality. The presence of residual cellular elements poses a significant risk of immunogenicity, potentially leading to immune rejection [6].

In 2015, Laronda et al. successfully decellularized bovine and human ovaries using SDS and subsequently recellularized them with primary ovarian cells. Their findings indicated that the decellularized scaffolds effectively preserved ovarian microstructure and facilitated estradiol hormone production in vitro [39]. Liu et al. implemented a combination of physical, chemical, and enzymatic treatments to decellularize porcine ovaries, aiming to reduce the duration of SDS exposure and mitigate its detrimental effects on tissue integrity. They found that their three-step decellularization protocol, which included Triton X-100, SDS, and DNase I, successfully removed cellular components while ensuring cell compatibility with minimal host immune responses, promoting cell penetration and enhancing estradiol production [40]. More recently, Alshaikh et al. explored the decellularization of mouse ovarian tissue employing two types of detergents: 0.5% SDS and 2% sodium deoxycholate. Although both methods exhibited high cell compatibility, the latter demonstrated slightly superior recellularization efficiency [41].

Eivazkhani et al. investigated the effects of two distinct treatments—SDS and sodium hydroxide (NaOH)—on the decellularization of mouse, sheep, and human ovaries. Their

results showed that scaffolds created using NaOH achieved better decellularization outcomes and supported cell growth more effectively than those treated with SDS [42].

Hassanpour et al. developed cytocompatible three-dimensional human decellularized ovarian scaffolds utilizing a sodium lauryl ether sulfate (SLES)-based protocol. They reseeded primary ovarian cells onto these scaffolds, resulting in grafts that were transplanted into rats. After a 14-day period, these grafts exhibited good cell compatibility, underscoring the material's potential for ovarian tissue reconstruction [43].

The use of detergent–enzymatic methods for decellularizing ovarian tissue, while commonly employed and capable of producing functional constructs, presents significant challenges. This multistep process is time-consuming and often alters the composition of the ECM, leading to reductions in critical components, such as GAGs and cytokines [34]. GAGs are critical to regulate processes from cell growth to cell migration. Cytokines, also regulate the overall immune function [44]. Consequently, the structural and mechanical properties of the resulting scaffolds may be compromised. In addition, the decellularized matrices must undergo sterilization, a step that can further impact their mechanical and biological characteristics [6, 45]. Decellularizing dense, poorly vascularized tissues like the ovary is particularly difficult due to the slow diffusion of detergents within the tissue. This slow penetration can result in residual detergent remaining in the tissue, which may exhibit cytotoxic effects, thereby impairing cell attachment and potentially inducing adverse reactions, such as inflammation and thrombus formation post-transplantation [36, 46]. To mitigate these issues, $scCO_2$ has emerged as a promising alternative method for decellularization. $scCO_2$ possesses unique physical and chemical properties, including high fluidity, excellent solubility, and superior heat transfer efficiency due to its density being close to that of a liquid while maintaining gas-like viscosity and diffusion characteristics [21]. By integrating $scCO_2$ with traditional decellularization processes, it is feasible to address the challenges associated with conventional methods effectively. Recent studies have demonstrated that the effectiveness of $scCO_2$-based decellularization has been demonstrated across various tissue types. In this study, we applied $scCO_2$ at 200 bar pressure and 40 °C for 1.5 h, with 1% SDS pretreatment for decellularization of human ovarian tissues. This contrasts with protocols used for other tissues, such as arteries (300 bar, 37 °C), porcine heart valves (250 bar, 37 °C), and cartilage (100 bar, 35 °C), showing that optimal $scCO_2$ parameters are tissue-specific. The unique structure and cellularity of human ovarian tissue required our modified approach to achieve effective decellularization while preserving ECM components [21]. Furthermore, $scCO_2$ can serve dual purposes by also acting as a sterilizing agent for decellularized scaffolds [47], thus streamlining the overall process while enhancing the quality of the resulting constructs. In summary, while traditional detergent–enzymatic methods for ovarian tissue decellularization have their merits, the introduction of $scCO_2$ presents a viable alternative that could improve both efficiency and outcomes in tissue engineering applications.

The use of $scCO_2$ for decellularization has been explored in other tissue types. Sawada et al. reported effective, though incomplete, decellularization of adipose tissue using $scCO_2$ at 20 MPa (200 bar) and 40 °C [33]. Consistent with their findings, Phase One of our experiment using $scCO_2$ at 200 bar and 40 °C also resulted in a significant reduction of DNA content, but did not fully meet the established decellularization

criteria. This similarity suggests that while $scCO_2$ alone at these parameters can disrupt cells and extract some cellular material, it may not be sufficient for complete decellularization, particularly in denser tissues like the ovary.

The efficacy and speed of the cell disruption process using $scCO_2$ are influenced by several factors, including the size, density, porosity, and composition of the tissue, particularly the levels of water and both polar and non-polar compounds. These factors are intricately linked to the unique architecture of cell membranes, which consist of a phospholipid bilayer. The amphipathic nature of phospholipids—having hydrophilic (water-attracting) heads that face outward and hydrophobic (water-repelling) tails that face inward—plays a crucial role in cell integrity. Similarly, nucleic acids exhibit a highly polar hydrophilic exterior and a hydrophobic core, which further complicates the disruption process [47]. To enhance the efficiency of tissue cell disruption during $scCO_2$ treatment, several strategies have been proposed to increase polarity within the protocol. While $scCO_2$ alone can disrupt cells and extract some cellular material, the studies of Han, Duarte, Antons, and others have demonstrated that the use of $scCO_2$ in combination with other techniques is beneficial for achieving the complete decellularization of tissue.

These pretreatments may involve physical or chemical methods that prepare the tissue for more effective $scCO_2$ penetration. In addition, utilizing cosolvents such as methanol or ethanol alongside CO_2 has been shown to enhance the solvation properties of $scCO_2$. These solvents can interact with the polar regions of cell membranes and nucleic acids, facilitating better disruption of cellular structures. By integrating these cosolvents into the $scCO_2$ protocol, researchers can potentially overcome some of the limitations associated with traditional decellularization methods [47].

Therefore, in the second phase, two modifications were implemented to identify the most optimal protocol for enhancing the decellularization process: the addition of 70% ethanol as a co-solvent and the application of 1% SDS as a pretreatment while utilizing the $scCO_2$ system under the previously established conditions. In samples treated with $scCO_2$ combined with ethanol, despite the transparent appearance of the tissue, insufficient reduction of DNA levels to the desired threshold was still observed in the DNA quantification test.

Antons et al. were the first to apply $scCO_2$ for decellularizing dense tissues, such as articular cartilage, tendon, and skin. Initial tests with $scCO_2$ alone did not reduce DNA levels, but the addition of LS-54, as a CO_2-philic detergent, resulted in significant reductions—approximately 67% for tendon and 60% for skin. However, this treatment was insufficient for cartilage, leading the authors to implement additional pretreatment steps, which achieved about 82% DNA reduction in cartilage and 95% in skin [48]. Duarte et al. also tested the use of TnBP as a cosolvent for $scCO_2$-mediated decellularization of trabecular bone, where there was a 70–90% DNA content reduction, but still presented with visible nuclear material after the treatment, thus indicating the potential for further optimization [49]. In the other study, Han et al. successfully achieved decellularization of the swim bladder in fish using the $scCO_2$ system at a temperature of 35 °C, a pressure of 250 bar, and a duration of 2 h, along with the pretreatment of SDS and the co-solvent ethanol at 70% [50].

In this study, we found that immersing the samples in SDS for 4 h followed by an overnight washing procedure and subsequent treatment with $scCO_2$ established an optimal protocol for preserving the essential characteristics of the ovarian ECM. Our data confirmed that this protocol is able to meet previously established decellularization criteria such as invisible nuclear material in the tissue sections and < 50 ng dsDNA/mg dry ECM weight [51]. Moreover, histological staining showed that the extracellular matrix structure and composition were satisfactorily preserved after decellularization. The SEM also confirmed the efficient removal of the cells and good preservation of three-dimensional structures and integrity after decellularization.

The ECM is primarily composed of collagens and GAGs, which play a crucial role in the biomechanical properties of tissues. GAGs, known for their high-water absorption capacity, occupy a significant portion of the extracellular space, providing mechanical support and facilitating the rapid diffusion of water-soluble molecules and cell migration. The preservation of GAGs is vital for promoting cell growth after reseeding onto decellularized scaffolds [52]. In this study, GAGs content was quantified, revealing a significant reduction compared to native tissue; however, more than 70% of the GAGs content was successfully preserved. This finding aligns with existing literature indicating that decellularization protocols using 1% SDS and Triton X-100 typically result in GAGs loss. While SDS is effective in removing nuclear materials from ovarian tissue, it can also lead to a decrease in GAGs levels within the tissue [53]. Furthermore, the cytocompatibility of $scCO_2$-based decellularized scaffolds was evaluated by assessing the viability and proliferation of human Wharton's jelly mesenchymal stem cells cultured on these scaffolds. The MTT assay demonstrated that cells remained viable and proliferated effectively within the scaffold. Notably, the proliferation rate on scaffolds during the first day was significantly lower compared to cells cultured in traditional two-dimensional environments, suggesting an adaptation period for cells adjusting to the three-dimensional culture environment and scaffold matrix [54]. However, by days 3 and 5, cell viability and proliferation on decellularized scaffolds surpassed those observed in two-dimensional cultures.

While our decellularization protocol effectively reduced DNA content and preserved ECM structures, it is important to consider the potential for residual components to elicit an immune response. As demonstrated by Padma et al. [35], decellularized scaffolds can contain damage-associated molecular patterns (DAMPs) that trigger inflammation and rejection [35]. Therefore, comprehensive safety evaluations, including assessments of immunogenicity and in vivo studies at multiple timepoints, are crucial for evaluating the biocompatibility and bioactivity of decellularized scaffolds. In addition, quantitative tensile strength testing is essential to comprehensively assess the biomechanical integrity of $scCO_2$-based ovarian decellularized scaffolds under physiological conditions.

Conclusion

Considering the adequate preservation of ECM structures, suitable cell compatibility, reduced processing time, and decreased reliance on chemicals and repetitive tissue washes for decellularization, we propose an optimal protocol utilizing 1% SDS as a pretreatment for 4 h, followed by the application of the $scCO_2$ system at 200 bar and 40 °C for 1.5 h. This protocol addresses common challenges associated with traditional

decellularization methods, such as lengthy processing times and potential toxicity from residual chemicals. By integrating a mild detergent pretreatment with $scCO_2$ processing, this approach not only streamlines the decellularization process but also minimizes adverse effects on cell viability and proliferation when reseeded on the scaffolds. Overall, this protocol presents a promising avenue for advancing ovarian tissue engineering applications.

Materials and methods

Ethical standards

This study was approved by the Ethics Committee of Shiraz University of Medical Sciences (registration number: IR.SUMS.REC.1402.095).

Human ovarian tissue collection

For the preparation of a cell-decellularizing scaffold, healthy human ovarian tissue was collected from transgender volunteers aged 18–35 and patients with endometriosis who underwent surgery, with informed consent obtained. The entire ovarian tissue was transferred to the Stem Cell Laboratory of the Anatomy Department at Shiraz Medical School in less than 2 h after being removed from the body, stored on ice in PBS solution. The samples were washed with PBS, and damaged areas with blood clots were removed.

Decellularization process

For decellularization, the ovaries were bisected along the hilus and cut into manageable strips. Then medulla separated from cortex to prepare ~ 2.0 mm thick tissue slices of cortex (Fig. 11).

In this study, building upon our previous research concerning rat testicular tissue, we designed four distinct experimental sets aimed at determining the optimal parameters for the decellularization of human ovaries utilizing $scCO_2$. Each experimental set varied in specific conditions, including pressure and treatment methods, to thoroughly evaluate their respective impacts (Table 1). The efficacy of each decellularization protocol was

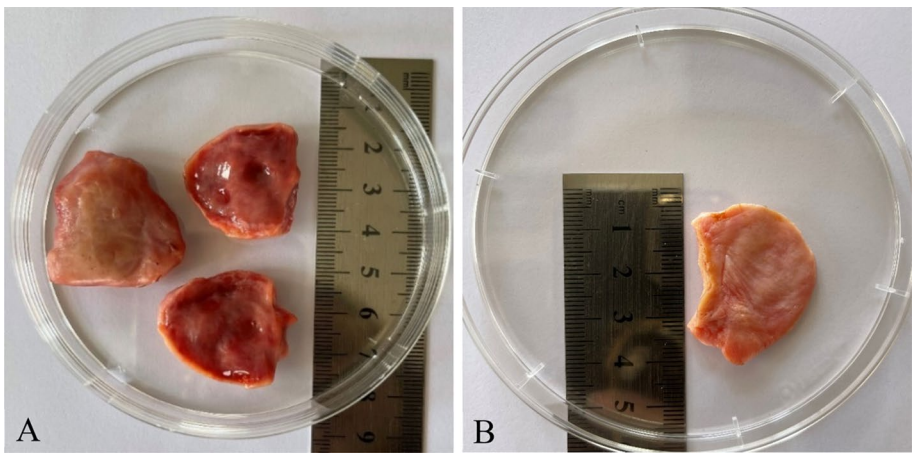

Fig. 11 For decellularization, the ovaries were bisected along the hilus (**A**), and the medulla was separated from the cortex to prepare approximately 2.0 mm thick tissue slices of the cortex (**B**)

Table 1 Experimental protocols

	Protocols	Extraction parameters	Co-solvent	Pretreatment
Phase 1	$scCO_2$	$T=40$ °C, $P=200$ Bar, $t=1.5$ h $T=40$ °C, $P=$, $t=1.5$ h	–	–
Phase 2	$scCO_2$ + Co-solvent	$T=40$ °C, $P=200$ Bar, $t=1.5$ h	Ethanol 70%	–
	Pretreatment + $scCO_2$	$T=40$ °C, $P=200$ Bar, $t=1.5$ h	–	$\boldsymbol{t}=4$ h

$scCO_2$ supercritical carbon dioxide, *SDS* sodium dodecyl sulfate, *T* temperature, *P* pressure, *t* time

assessed through H&E staining and DNA quantification tests conducted on both native and decellularized tissues. For each experimental set, approximately 1 × 1 cm pieces of wet ovarian slices were utilized, with samples being meticulously loaded into a tissue holder and carefully positioned within the $scCO_2$ system.

At the first phase of experiment (evaluation of pressure), the $scCO_2$ system was used at temperatures of 40 °C and pressures of 200–300 bar for 1.5 h. Initial assessments of the decellularization process's success were conducted. The H&E staining and DNA quantification results showed that the phase one decellularization protocol was insufficient. In the second phase, two modifications were used to find the most optimal protocol to improve the decellularization process. According to previous literatures, adding 70% ethanol as a co-solvent or 1% SDS as a pretreatment improves the effectiveness of $scCO_2$ (Table 1) [25].

To evaluate the potential of a cosolvent to enhance decellularization, a subset of ovarian tissue samples was treated using a protocol referred to as "$scCO_2$ + cosolvent." In this protocol, ovarian tissues were placed in the $scCO_2$ vessel, ensuring they did not directly contact the 70% ethanol, which occupied approximately 6% of the vessel volume. Subsequently, the system was operated for 1.5 h at 200 bar and 40 °C in static mode.

In the pretreatment + $scCO_2$ protocol, ovarian tissues are first placed in 1% SDS on a magnetic stirrer for 4 h at room temperature. Then, samples are washed overnight in sterile PBS. Subsequently, they are loaded into the tissue holder of the device for 1.5 h under conditions of 200 bar and 40 ℃. In addition, some ovarian tissues treated with SDS alone for 4 h are designated as the control group.

DNA content analysis

To assess the amount of DNA in both native and decellularized ovarian tissue, the DNA Quantification technique was employed following the protocol provided in the KiyanEX one-step total DNA extraction kit and the DNA yield (ng/μL) was quantified spectrophotometrically (the OD at $\gamma=260$ nm), using the NanoDrop® ND-1000 (Nanodrop Technologies Inc., Wilmington, DE, USA.

Histological assessment

Histological evaluations were initiated by fixing the decellularized scaffolds and native tissues in formalin. After basic tissue processing, the samples were embedded in paraffin and sectioned 5–10 μm thickness. For confirmation of efficient cell removal and evaluation of overall tissue architectural, the sections were stained with H&E.

Extra cellular matrix structures were qualitatively evaluated by the Masson Trichrome staining for collagen fibers, Alcian blue (pH 2.5) for glycosaminoglycan, Gomori's aldehyde–fuchsin for elastic fibers and PAS to evaluate neutral sugars.

SEM

To assess the ultrastructure of decellularized ovarian scaffolds, imaging was performed using SEM. The lyophilized samples were coated with gold using a Sputter Coater (Q150 R-ES, Quorum Technologies, UK) in the central laboratory of Shiraz University. Finally, the samples were observed and imaged using a VEGA3 electron microscope (TESCAN, Czech Republic).

GAGs content analysis

To evaluate residual GAGs approximately 100 mg of the lyophilized powder of each decellularized and native tissue was hydrolyzed in 0.25 mL of 6 M hydrochloric acid at 95 °C for 20 h. After cooling, 20 µL of each sample was mixed with 200 µL of 1,9-dimethylmethylene blue in a microplate, and OD was measured at 656 nm using a microplate reader. The GAGs concentrations were then determined using a calibration curve prepared with heparin serial dilutions. Standard dilutions of heparin were prepared through a six-step dilution process, and the resulting ODs were used to plot the standard curve for GAGs concentration calculation.

Cytotoxicity assay

At the end of the decellularization, the prepared scaffolds are lyophilized and then sterilized with UV light exposure under a sterile hood. To assess scaffold's cytocompatibility, HWJMSCs were loaded at a density of 150,000 cells onto each scaffold and cultured under standard conditions (5% CO_2, 37 °C). The same number of HWJMSCs was seeded in a conventional two-dimensional condition as the control culture. Cell viability and proliferation were assessed based on a previously described MTT method used by Hassanpour et al. [43] on days 1, 3, and 5 of culturing.

Statistical analysis

Quantitative data were expressed as the mean value ± standard error of mean (S E M). Independent samples *t* test for DNA quantification, two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test for MTT assays' data, and one-way ANOVA followed by Tukey's multiple comparison test for GAGs assays' data were performed. The data were analyzed using GraphPad prism 10 software. P values < 0.05 were considered as significant.

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Author contributions
F H performed the experiments and was involved in the collection, analysis and interpretation of data, and manuscript drafting. A H and A Z H. conceived the original idea and supervised the project. A H, TTK and A Z H interpreted the data and revised the manuscript. M R assisted in data collection. All authors read and approved the final manuscript.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This study was approved by the Ethics Committee of Shiraz University of Medical Sciences (Registration Number: IR.SUMS.REC.1402.095).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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