Decellularization Techniques to Manufacture Ovary Cancer Niche Scaffold as Prototype for 3D Cancer Cell Culture

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Abstract. Ovarian cancer contains a microenvironment that supports the proliferation of cancer cells. The microenvironment of ovarian cancer can be produced in vitro by the manufacture of an ovarian cancer scaffold. The scaffold provides conditions structurally similar to the in vivo microenvironment for communication between cells and cells with the extracellular matrix (ECM) thus providing in vitro platform for disease modeling or drug screening. The decellularization method is used to remove cellular components from the ECM with little damage to the structure. The manufacture of ovarian cancer scaffolds in this study are carried out by physical and chemical decellularization techniques with comparison between one, two or three cycles of freeze-thawing. The process of making an ovarian cancer scaffold is using NaOH 0.4N with one, two or three times of freeze-thawing (−80 °C and 37 °C) followed by agitation with an orbital shaker. Characterization of the scaffold was performed by measurement of DNA concentration, histology analysis of decellularization by hematoxylin eosin and scanning electron microscopy (SEM), ECM analysis by masson trichrome for collagen area fraction, collagen III immunohistochemistry, fibronectin immunohistochemistry and byglican immunohistochemistry, cytotoxicity assay of SKOV-3 cell line using flow cytometry 7-AAD. The average genetic material of DNA from the scaffold is lowest at 4.1 ng/µl from the three times freeze-thawed cycles. Hematoxylin-Eosin staining showed a minimum amount of absence of nuclei and presence of intact ECM structure in fibers form particularly in the three times freeze-thawed

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cycles. SEM result confirmed absence of nuclei and preservation of ECM fibrous structure at the surface of the scaffold in the three times freeze-thawed cycles. The integrity of ECM is preserved with minimal damage to the ECM showed in three times freeze-thawed cycles. Masson's Trichrome staining showed the collagen fibers in ECM had less damage and more collagen consistently in the three times freeze-thawed cycles. Immunohistochemistry of the ovary cancer scaffold showed the presence of collagen type III, fibronectin, and biglycan with consistently similar area fraction between three times freeze-thawed cycles and non decellularized ovarian cancer tissue which indicate preservation of those ECM proteins. The ovary cancer scaffold displayed very low toxicity to SKOV-3 cell line. The ovarian cancer scaffold manufactured in the three times freeze-thawed cycles has the least DNA, more intact collagen fibers, preserved ECM proteins e.g. collagen type III, fibronectin, and biglycan, and very low toxicity to SKOV-3 cell line.

Keywords: ovarian cancer · scaffold · decellularization · freeze-thawing · repeated cycles

1 Introduction

Ovarian cancer is the malignant and deadliest tumor that threatens women. According to the World Health Organization (WHO), global cases of ovarian cancer in 2018 were recorded at 295,414 [1]. Ovarian cancers are often diagnosed too late due to the absence of specific initial symptoms and the lack of effective screening for early detection results in an advanced diagnosis [2]. Advanced ovarian cancer has a high morbidity and mortality. Ovarian cancer contains a microenvironment to support the proliferation of cancer cells. The microenvironment of cancer (niche) consists of several cells, blood vessels, soluble factors and the extracellular matrix (ECM). ECM consists of collagen, proteoglycans in the form of glycosaminoglycans (GAG), laminins, fibronectins, and elastin [3]. Cancer blood vessels are important for cancer cell activity. ECM and cancer vessels are present in the cancer niche to facilitate cancer cell proliferation, metabolism, angiogenesis and metastasis [4, 5]. An extracellular matrix (ECM) is a complex network consisting of an arrangement of macromolecules arranged specifically by cells/tissues. ECM components are connected together to form a structurally stable composite thus contributing to the mechanical properties of the network. The ECM is a reservoir of growth factors and bioactive molecules thus determines the behavior and characteristics of cancer cells. Cancer niche produce immunosuppressive conditions. New innovative treatments for ovarian cancer using immunotherapy will need 3D culture in the cancer niche. 3D culture in cancer niche can be reconstructed in vitro with production of ovarian cancer scaffold. Scaffold provides conditions structurally similar to the in vivo microenvironment for communication between cells and cells with extracellular matrix (ECM) [6].

Decellularization is one of techniques to manufacture native scaffold [7]. The decellularization method can be done physically, enzymatically and chemically. The objective of decellularization is to remove cellular components from the tissue with little damage to the ECM structure. After decellularization, the remaining components are the ECM and the blood vessels. The technique of physical decellularization consists of freeze-thawing and agitation [4]. The most commonly used physical technique for the manufacture of ovarian scaffold is freeze-thawing. Freeze-thawing forms intracellular ice crystals, disrupts cell membranes and causes cell lysis. Another physical decellularization technique is by mechanical agitation using orbital shakers. Chemical decellularization technique can be combined with physical decellularization technique to increase cell lysis and cellular debris removal [8]. Chemical decellularization can be done by using chemical compounds (NaOH) or detergents. Detergents are chemicals for dissolving cell membranes [4, 8].

The integrity of the ECM components is prone to damage due to decellularization. This research is comparing decellularization techniques. This research was conducted to analyze the effect of differences in freeze-thawing to the ECM component of the scaffold. It helps to find the decellularization technique that produces ovary cancer scaffolds with less damage to ECM and has great protein in ECM.

2 Materials and Methods

2.1 Ovarian Tissue Decellularization Techniques

- Set 1: Ovarian cancer tissue is cut by $0.5 \times 0.5 \times 0.5$ cm^3. The decellularization process is carried out with 1 cycle of freeze-thawing at −80 °C and 37 °C. The sample was incubated in NaOH (0.4 N) 48 h in an orbital shaker. All tissues were washed with sterile PBS for 48 h with an exchange of at least 6 times to remove cell debris and reagents.
- Set 2: Same as set 1except for 2 cycles of freeze-thawing at −80 °C and 37 °C
- Set 3: Same as set 1 except for 3 cycles of freeze-thawing at −80 °C and 37 °C

2.2 Characterization of Ovarian Cancer Scaffolds

2.2.1 Measurement of Scaffolding Genetic Material

The extraction process is carried out with a G-Spin DNA isolation kit. DNA quantification was performed by spectrophotometry to measure optical density (OD) at a wavelength of 260 nm.

2.2.2 Morphological Analysis of Scaffold

The sample was fixed in formaldehyde 10% and conducted tissue preparation with dehydration in the alcohol series. The sample is planted in paraffin and then cut by 5 μm. The sample was colored Hematoxylin and Eosin (HE), Masson's Trichrome (MT). Sample were examined with a light microscope and photographed with optilab.

2.2.3 Ultrastructure of the Extracellular Matrix

The block paraffin tissue was sent to CMPFA, Depok, Universitas Indonesia. The morphology and structure of the sample were analyzed using a Scanning Electron Microscope (SEM).

2.2.4 Extracellular Matrix Components Analysis

Immunohistochemistry collagen 3, fibronectin, and biglycan are performed to evaluate the integrity of the extracellular matrix. Rabbit polyclonal primary antibodies (Abcam antibodies, ab7778, concentration: 1:500), rabbit polyclonal primary antibodies (Abcam atntibodies, ab23751, concentration: 1:750) and mouse monoclonal primary antibodies (antibodies Santa Cruz, sc-100857, concentration: 1:1000) used for the assessment of type 3 collagen, fibronectin, and biglycan. Preparations are carried out of deparafination. Endogenous peroxidase was blocked with hydrogen peroxide block. Non-specific protein activity is then blocked with protein blocks. The sample was incubated with primary antibodies at a temperature of 4 °C overnight. The next day, samples were incubated with HRP-conjugated secondary antibodies for 1 h. The sample is added with 3-3'-diaminobenzidine (DAB). Samples were counterstained with hematoxylin and lithium carbonate. Samples were dehydrated with alcohol series. Samples were soaked in xylol and closed with entelan. Preparations are examined with a light microscope and photographed with an optilab. Measurement of the optical density and area fraction compared between the sample and the control (non-decellularized tissue) with imageJ [9, 10].

2.2.5 Flow Cytometry 7-AAD Analysis after 48 h SKOV-3 Culture on the Scaffold

Cytotoxicity assay was carried out by cell line culture SKOV-3 on the scaffold for 48 h followed by trypsinization and harvest. The harvested cells were incubated with 7-AAD for 30 min and washed by centrifugation. Flow cytometry of 7-AAD was performed to investigate the number of dead cells as the dye intercalated with DNA of dead cells. The lower the percentage of 7-AAD positive cells indicate low toxicity level of scaffold toward the cultured cells. Non decellularized ovary cancer tissue piece was used as control.

3 Results

3.1 Material Genetic (DNA)

Haematoxylin-Eosin staining showed that there was minimal nucleus in the scaffold compared to control. The structure of ECM (fibers), blood vessels (capillaries) that are more intact and not disrupted is in Set 3. DNA concentration in Set 3has the least DNA compared to other groups. Set 3 with freeze-thawed 3 cycles resulted in scaffolds that have least DNA concentration and intact structure of the scaffold. Set 1, Set 3 and Set 3 have no significant differences compared to each other. But, all groups have significant differences with control. Haematoxylin Eosin staining and DNA concentration shown in Fig. 1.

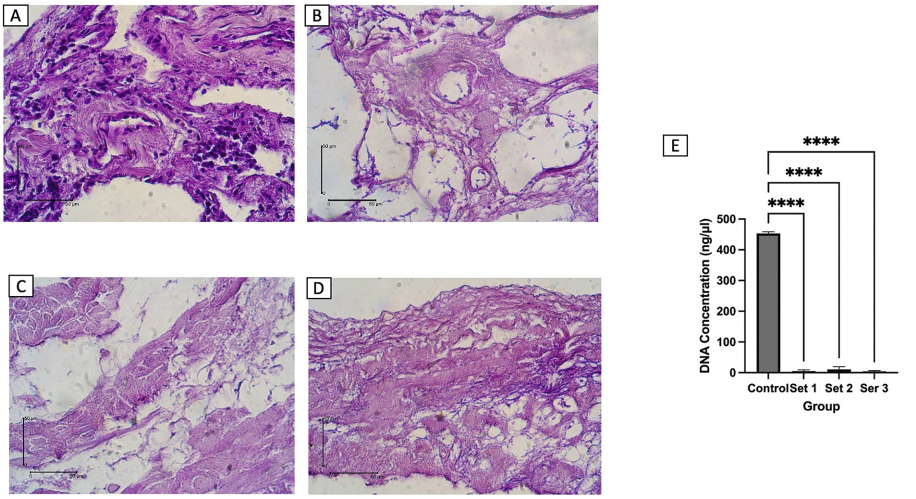

Fig. 1. The Haematoxylin Eosin staining and DNA concentration. A: HE of ovary cancer non-decellularization (control); B; HE of Set 1 scaffold; C: HE of Set 2 scaffold; D: HE of Set 3 Scaffold; E: DNA concentration. HE: Haematoxylin-Eosin; Set 1: 1 freeze-thawing cycle; Set 2: 2 freeze-thawing cycles; Set 3: 3 freeze-thawing cycles.

3.2 Ultrastructure Scaffold Using SEM

Ultrastructure in the scaffold showed that Set 3 has a better surface compared to other groups. Set 3 also showed that there are no more cells observed. Meanwhile, Set 1 has more cells observed. Set 2 has a frown surface of the scaffold. Ultrastructure of the scaffolds using SEM shown in Fig. 2.

3.3 ECM Protein Composition: Collagen

Collagen plays a role in maintaining tissue integrity. Collagen in scaffold analyzed by Masson's Trichrome staining and Immunohistochemistry. Masson's Trichrome staining showed that Set 3 has more collagen than other groups. Collagen stained in blue color. The structure of the scaffold in Set 3 is also more intact than other groups. Immunohistochemistry of collagen showed that area fraction and optical density of collagen is higher than other groups. But, there are no significant differences between all the groups. Masson's Trichrome staining, Immunohistochemistry and Image J analysis shown in Fig. 3.

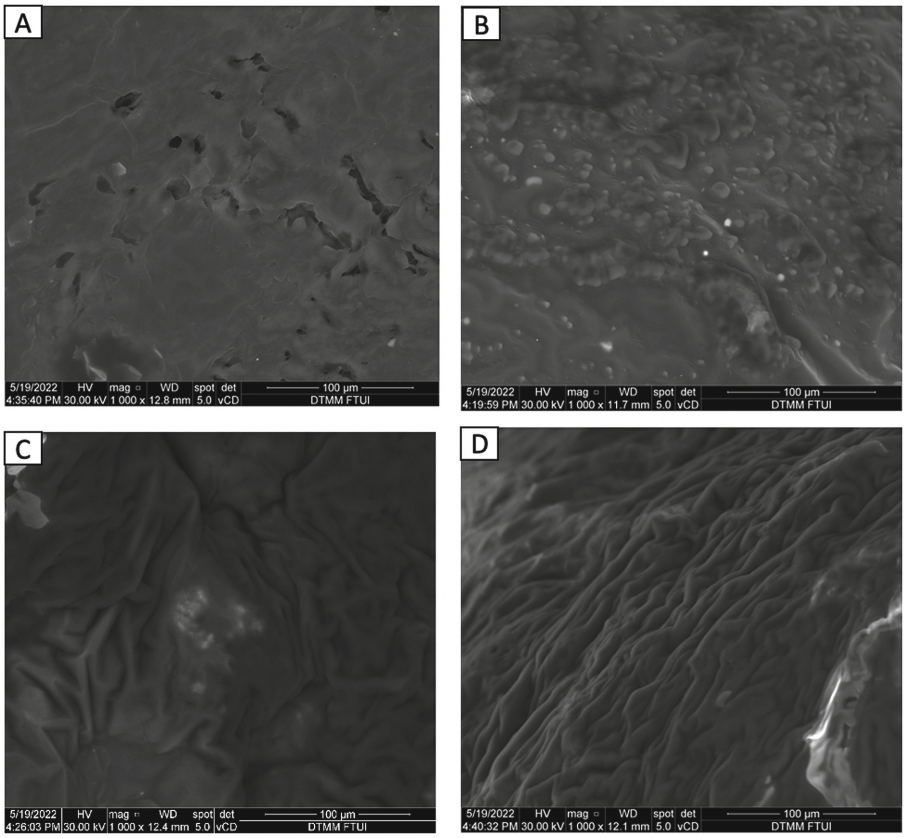

Fig. 2. Ultrastructure of the scaffolds. A: SEM of control; B: SEM of Set 1 Scaffold; C: SEM of Set 2 Scaffold; D: SEM of Set 3 Scaffold. SEM: Scanning Electron Microscope; Set 1: 1 freeze-thawing cycle; Set 2: 2 freeze-thawing cycles; Set 3: 3 freeze-thawing cycles.

3.4 ECM Protein Fibronectin

Fibronectin plays a role in cell adhesion. Fibronectin in scaffolds analyzed by Immunohistochemistry showed that area fraction and optical density of biglycan is higher than other groups. Optical density of set 3 is significant compared to all the groups and control. But, area fraction of all the groups are no significant differences. Immunohistochemistry and Image J analysis shown in Fig. 4.

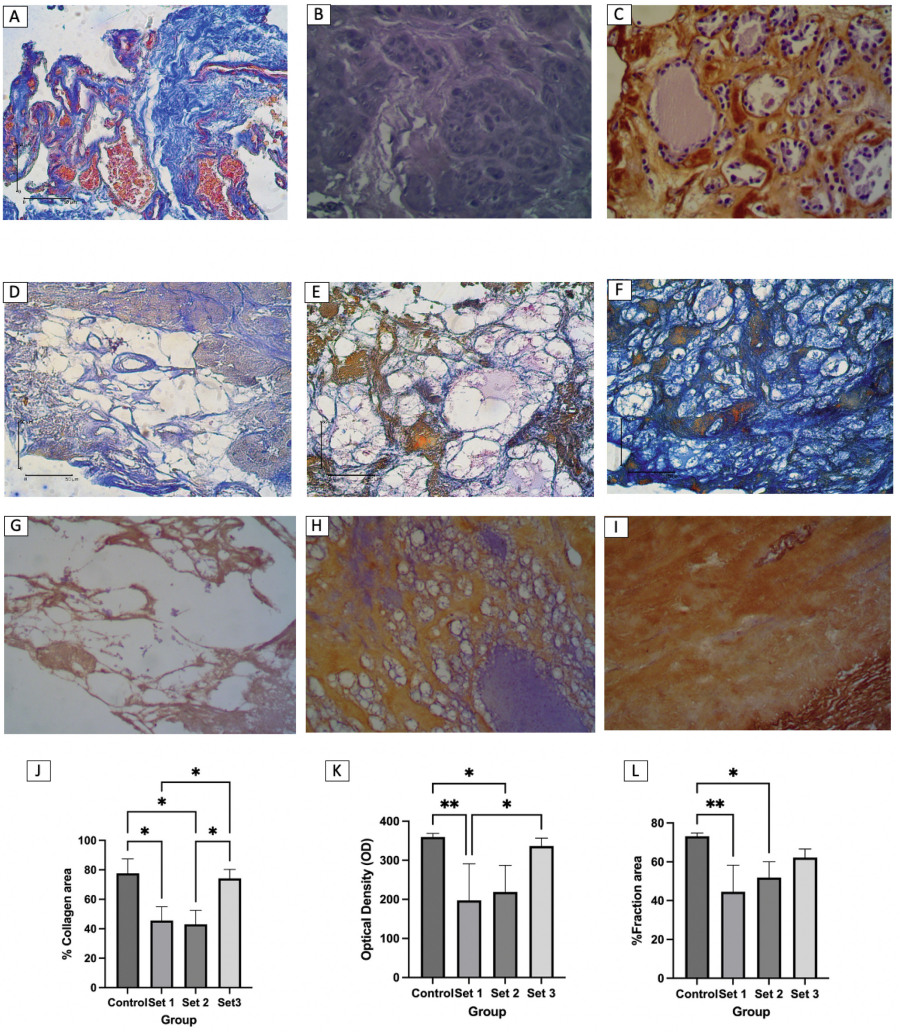

Fig. 3. Masson's Trichrome staining, Immunohistochemistry (IHC) of collagen and Image J analysis. A: MT of control; B: IHC of control negative; C: IHC of control positive; D: MT of Set 1 Scaffold; E: MT of Set 2 Scaffold; F: MT of Set 3 Scaffold; G: IHC of Set 1 Scaffold; H: IHC of Set 2 Scaffold; I: IHC of Set 3 Scaffold; J: collagen area; K: optical density; L: fraction area. MT: Masson's Trichrome; IHC: immunohistochemistry; control: ovary cancer non-decellularization; control negative: IHC of ovary cancer non-decellularization with no primary antibody collagen; control positive: IHC of ovary cancer non-decellularization with primary antibody collagen; Set 1: 1 freeze-thawing cycle; Set 2: 2 freeze-thawing cycles; Set 3: 3 freeze-thawing cycles.

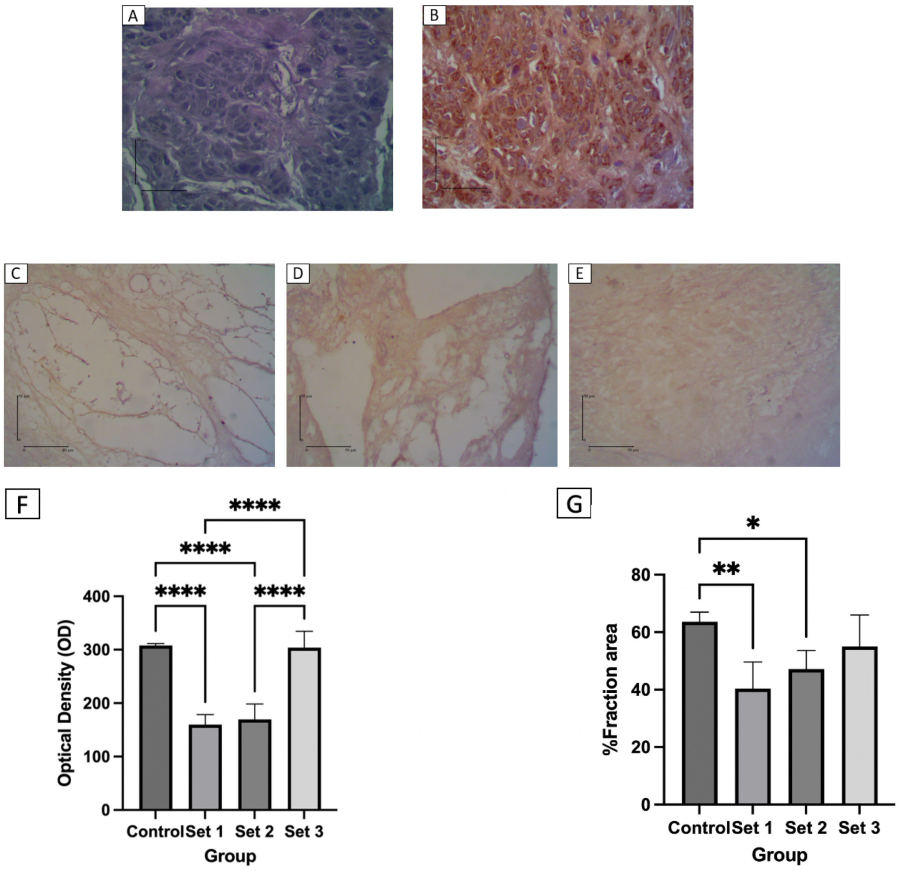

Fig. 4. Immunohistochemistry (IHC) of fibronectin and Image J analysis. A: IHC of control negative; B: IHC of control positive; C: IHC of Set 1 Scaffold; D: IHC of Set 2 Scaffold; E: IHC of Set 3 Scaffold; F: optical density; G: fraction area. IHC: immunohistochemistry; control: ovary cancer non-decellularization; control negative: IHC of ovary cancer non-decellularization with no primary antibody collagen; control positive: IHC of ovary cancer non-decellularization with primary antibody collagen; Set 1: 1 freeze-thawing cycle; Set 2: 2 freeze-thawing cycles; Set 3: 3 freeze-thawing cycles.

3.5 Biglycan

Biglycan play a role in stiffness of tissue structure. Byglican in scaffolds analyzed by Immunohistochemistry showed that area fraction and optical density of biglycan is higher than other groups. But, area fraction and optical density of all the groups are no significant differences. Immunohistochemistry and Image J analysis shown in Fig. 5.

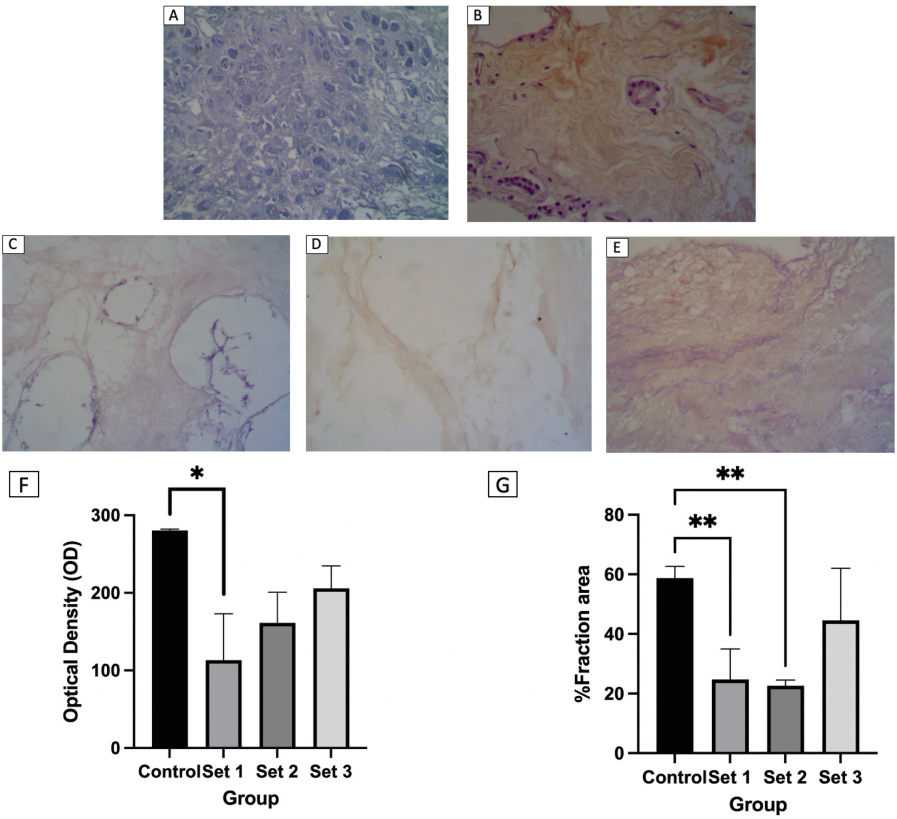

Fig. 5. Immunohistochemistry (IHC) of biglycan and Image J analysis. A: IHC of control negative; B: IHC of control positive; C: IHC of Set 1 Scaffold; D: IHC of Set 2 Scaffold; E: IHC of Set 3 Scaffold; F: optical density; G: fraction area. IHC: immunohistochemistry; control: ovary cancer non-decellularization; control negative: IHC of ovary cancer non-decellularization with no primary antibody collagen; control positive: IHC of ovary cancer non-decellularization with primary antibody collagen; Set 1: 1 freeze-thawing cycle; Set 2: 2 freeze-thawing cycles; Set 3: 3 freeze-thawing cycles.

3.6 Cytotoxicity Assay

Percentage of dead cells in control is 2.7%. Triplication of cytotoxicity assay results from SKOV-3 cell line culture on scaffold showed the percentage of 7-AAD positive cells ranges between 4.5% to 7.4% (Fig. 6).

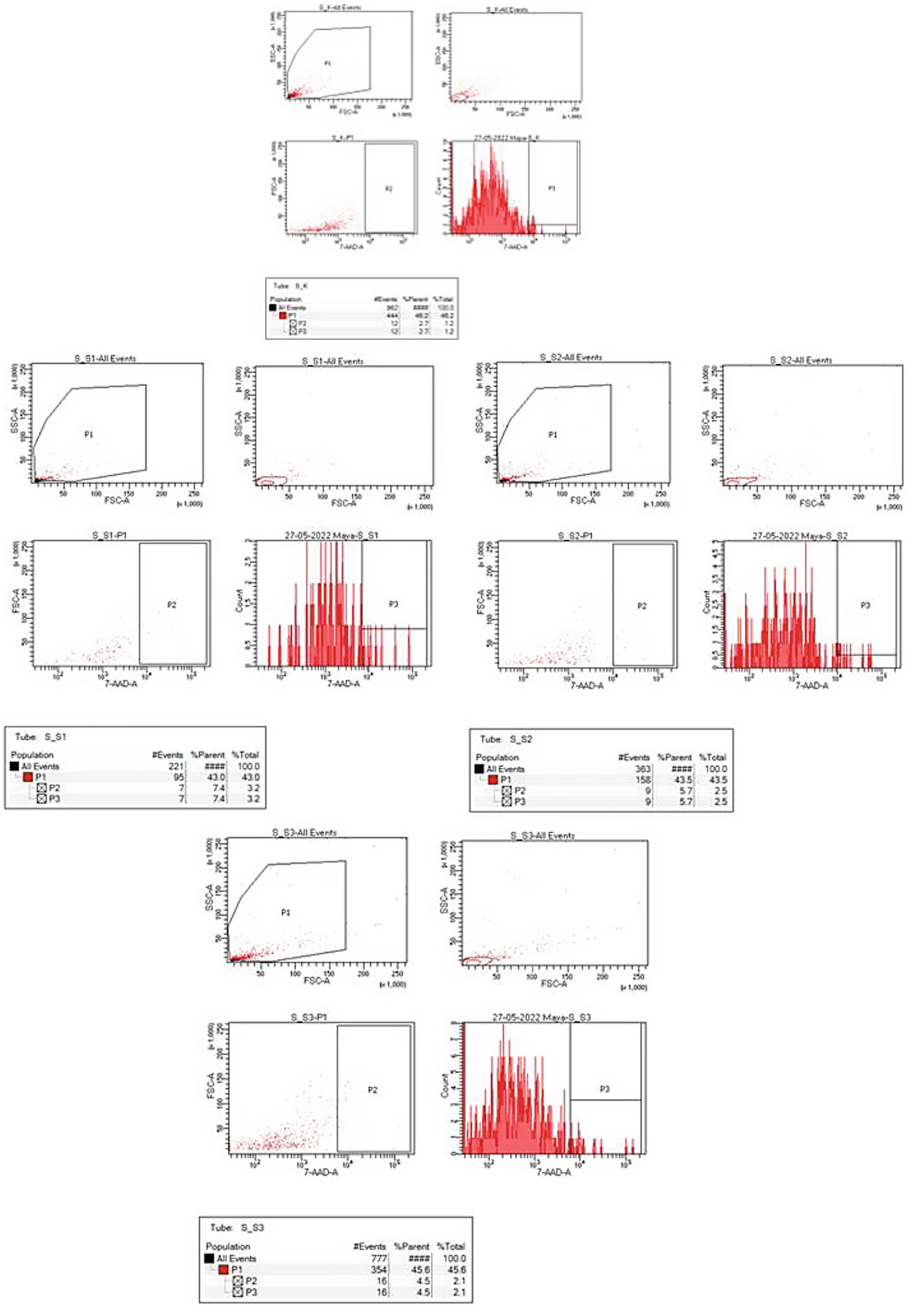

Fig. 6. 7-AAD flow cytometry result of control (upper centre graph) and 7-AAD flow cytometry result from optimized decellularization technique in triplication (row 2 and 3). Gating strategy was showed in the first dot plot. The percentage of 7-AAD positive cells is the percentage of P2 (dot plot) or P3 (histogram).

4 Discussion

Biological scaffolds that have similarity to the in vivo microenvironment recommended to use in cancer niche research. The results of this study indicate the manufacture of ovarian cancer scaffolds with combination of physical and chemical decellularization gives the best results in the cycle freeze-thawing 3x followed by agitation in NaOH on orbital shaker for 48 h and washing the scaffold with PBS for 6x changes in the above 48 h orbital shaker. The results of the decellularization process with 3x freeze-thawing scaffolds are transluscent clear. According to the study of Faulk et al. [11] after decellularization, tissues usually have a pale or translucent color. The decellularization affects cell removal from the tissue with little damage to the ECM. This research showed that material genetic analysis in 3x freeze-thawing scaffold has least DNA with absence of nuclei. The efficacy of cell removal is about 99.1% with remaining DNA concentration of 4,1 ± 2,536 ng/μl. This character fullfills criteria of ideal scaffold with less than 50 ng/μl DNA left on the decellularized scaffold [11, 12].

Freeze-thawing has been recognized as the initial step in decellularization technique which required combination with other technique. The mechanism of cell lysis by freeze thawing method is through the formation of intra and extracellular ice crystals that disrupts the cell membrane [13]. It induces thermal shock which lead to cell death. Combination with NaOH in this study further increase the efficacy for cell lysis. NaOH breaks down the cell wall and disrupts the hydrogen bonds in DNA. NaOH is the most efficient in terms of cell removal and cell viability after recellularization [14].

Repeated cycles of freeze-thawing have pros and cons in maintaining ECM integrity despite the effective cell removal [15, 16]. In this study, 3x freeze-thawing scaffold or the most frequent repeat of freeze thawing cycles has higher components of collagen, biglycan, and fibronectin than 1x or 2x repeated cycles. The results of combination NaOH and physical treatment of freeze-thawing showed there is no significant difference in reduction of biglycan, collagen, and fibronectin compared to control [14]. Fibronectin, collagen and biglycan were dispersed in ECM. Ultrastructure surface of the scaffold also confirms the absence of nuclei and denser fibrous structure at ECM surface of 3x freeze-thawing scaffold.

The reductions in the density and size of the scaffold after the decellularization caused problems. These problems caused not allowing the scaffold to further assays and application. The success in decellularization depends on density, thickness and cellularity of the tissues. It is important to maintain the structure, strands, and macromolecules of the tissue after decellularization [14]. 3x freeze-thawing scaffold has shown promising results with more intact scaffold and has the least reduction density of collagen, fibronectin and biglycan.

Arrangement and composition of collagens, proteoglycans, laminin, and fibronectin determine the structure and organization of the ECM. The components are made of special compositional and topographical features that both reflect and facilitate the functional necessities of the tissue [14]. Topographical features of SEM analysis in set 3 have a better surface scaffold than other groups.

SKOV-3 ovary cancer cell line can be cultured with the decellularized scaffold with very low toxicity than reported previously by Paradiso et al. [17]. This study showed cytotoxicity at a level of up to 7.4% while Paradiso et al. study indicated up to 14%

of cell death with the synthetic scaffold. The toxicity of scaffold in this study could be caused by traces of chemicals used in decellularization (NaOH) or sterilization (70% alcohol) technique.

Limitation of this research is the quantitative method of measurement ECM components is only using image analysis from immunohistochemistry result. Other quantitative method can be used to confirm measurement of ECM components e.g. ELISA kit, spectrophotometry, or combination of histology analysis with stereology.

5 Conclusion

The ovarian cancer scaffold decellularized in the three times freeze-thawed cycles followed by agitation in NaOH on orbital shaker for 48 h and washing the scaffold with PBS for 6x changes in the above 48 h orbital shaker have the least DNA, intact ECM (collagen type III, fibronectin, and biglycan), denser collagen fibers and very low toxicity.

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