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Design of a decellularized fish skin as a biological scaffold for skin tissue regeneration

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ABSTRACT

The use of decellularized natural skin as an extracellular matrix (ECM) may be a great candidate to regenerate damaged tissues. In this study, decellularized scaffolds from fish skin were designed by different techniques (physical, chemical, and enzymatic methods) and investigated by analyses such as Differential Scanning Calorimetry (DSC), Scanning Electron Microscopy (SEM), Tensile strength, Degradability, Histological studies, Toxicity test, and Determination of DNA content. Results showed that the best sample is related to the decellularized skin by hypertonic & hypotonic technique and Triton X100 solutions. Structural and mechanical results were demonstrated that samples have similar properties to human skin to regenerate it. The cytotoxicity results showed that decellularized skin by hypertonic & hypotonic method and Triton solution is non-toxic with minimal amount of genetic materials. Cellular results with epithelial cells indicated good adhesion on decellularized matrix, so it can be a suitable candidate for skin tissue regeneration.

1. Introduction

Biological scaffolds have been increasingly used to enhance the regeneration process in many reconstructive surgery procedures for more than a decade (Kumar et al., 2013a). However, the challenge facing tissue engineering is to combine different reagents to maintain the structure and function of the complex mixture of proteins that make up the extracellular matrix (ECM) (Kumar et al., 2012, 2013b). ECM is highly conserved among many species (Kumar et al., 2013c, 2013d). It is composed of molecules such as collagen, fibronectin, laminin, vitronectin, glycosaminoglycans (GAG) and growth factors, which are in a specific three-dimensional structure and the optimal composition for each source tissue (Kumar et al., 2013e; Courtman et al., 1994). Collagen is the main element of the mechanical structure of the dermis, which gives the tissue tensile strength and proteolytic resistance (Liang et al., 2004; Courtman et al., 2001). The ECM, which is composed of a complex network of structural and functional proteins such as collagen, can be used as a scaffold to support cell proliferation and ultimately tissue regeneration (Greco et al., 2015). The ECM molecules directly or indirectly signal cells to stimulate biological events and help a set of events to expand the distribution and differentiation of host-derived cells, respectively. Although the mechanisms of ECM in tissue regeneration have been not fully understood, new extracellular matrix regeneration is thought to occur from the release of bioactive peptides resulting from ECM degradation and stem cell signaling (Kulig et al., 2013; Reing et al., 2010; Chen et al., 2004; Crapo et al., 2011). The cells and the ECM are in a state of dynamic symmetry. This means that the cells respond to ECM signals to change their behavior, and the cells, in turn, change the structure of the ECM by changing their behavior. The architecture and composition of the ECM is unique to each tissue so it determines the function of the tissue (Gilbert et al., 2006; Badylak et al., 2009, 2011; Scarritt et al., 2015; Gilpin and Yang, 2017). Many synthetic biomaterials have been designed to regenerate damaged skin tissues (Biazar and Heidari Keshel, 2013, 2014; Biazar et al., 2014; Keshel et al., 2014; Biazar, 2016, 2017). Natural biomaterials extracted from plants and animals extra cellular matrices can be used as a

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substitute or scaffold in tissue regeneration tissue process (Rana et al., 2017; Keane et al., 2016; Hussein et al., 2016; Gilbert, 2012; Kwon and Moon, 2018; Zheng et al., 2018; Hussey et al., 2018; Mayorca-Guiliani et al., 2017). Natural scaffolds can be obtained by detoxifying tissues or organs while preserving the natural architecture and culturing one's own living cells on it to create natural tissue with the same natural function (Gilbert et al., 2006). To prevent this reaction of the immune system and achieve an extracellular matrix of tissue to cultivate the cells and thus produce new tissue that does not activate the immune system, must be done through various chemical, enzymatic, Physical and mechanical methods, and even a combination of these methods were used for decellularization. Use of these methods create the extracellular matrix as well as the three-dimensional architecture of the primary matrix to achieve a biological scaffold with a preserved structure (Scarritt et al., 2015; Gilpin and Yang, 2017). Biological materials from both allogeneic and xenogeneic tissue sources have been used to construct extracellular matrices for tissue regeneration. However, the prevalence of bovine spongiform encephalopathy and snow fever has limited the use of extracellular matrix (ECM) of bovine or porcine origin (Lau et al., 2019). Recently, due to religious restrictions and the risk of zoonotic disease among mammalian species, there has been interest in decellularized tissues from non-mammalian sources. In this regard, thanks to the structural and functional similarities of ECM between marine and mammalian tissues, the use of detoxified tissues of marine origin is considered. Fish do not contain prions or viruses that can be transmitted to humans (Kjartansson et al., 2015; Rakers et al., 2010; Magnusson et al., 2017; Dorweiler et al., 2017). Fish skin is a multifunctional tissue that performs many vital functions, including chemical and physical protection, sensory activity, behavioral goals, or hormone metabolism. In addition, it is an important first-line defense system against pathogens, as fish are constantly exposed to numerous microbial challenges in their aquatic habitats (Rakers et al., 2010). Fish skin is excellent in terms of highly antimicrobial properties that can be used as human skin. Mammalian scaffolds require extensive chemical processing to reduce the risk of virus and prion transmission, but scaffolds from fish skin undergo a gentle process to maintain the structure and composition of bioactive compounds, including omega-3 unsaturated fatty acids. Studies have shown that omega-3 fatty acids have antiviral and antibacterial properties and also act as inflammation regulators (Huang and Ebersole, 2010; Magnusson et al., 2017; Dorweiler et al., 2017). Clinical trials have shown that wound healing with fish skin results in faster wound closure than in mammalian matrices (Patel and Lantis, 2019). In this study, scaffolds from fish skin were decellularized by different techniques (Physical, chemical, and enzymatic methods) and studied by analyses such as Differential Scanning Calorimetry (DSC), Scanning Electron Microscopy (SEM), Tensile Strength, Degradability, Histological studies, Toxicity test, determination of DNA content, and also cell culture.

2. Materials and methods

Fresh Grass Carp skin (Tonekabon, Iran) was washed with cold water and descaled, then the skin was cut into 5 × 5 cm^2. Skin sections were washed with phosphate buffer solution (PBS), then frozen three times at −20 °C for 30 min and thawed at ambient temperature. The fish skins were washed again with phosphate buffer solution (PBS) and placed in hypertonic solution for 24 h. This solution contains a concentration of 1 M sodium chloride and 50 mM tris (Hydroxylmethyl) aminomethane and 10 mM ethylene di-amine tetra-acetic acid (EDTA) in PBS solution. They were washed with PBS and cold distilled water after exposure to hypertonic saline. Then, according to the following protocols and according to the table, several different protocols were performed to create decellularized scaffolds. Thickness of the samples after decellularization process was about 0.25 mm (Table 1 and Fig. 1).

2.1. Toxicity assay

Regarding to previous methods (Zeinali et al., 2014), proliferation of cells was determined from measurement of viable cell numbers by MTT assay. To assess cell viability and proliferation rate, C619 (Skin BALB/c mouse derived; NCBI Code: C619) epithelial cell line (Iranian pasture institute cell bank) was used. In brief, the cells were plated in 96-well culture plates at a density of 1 × 10^4 cells/well. Pieces measuring 3 mm in the shape of a circle were cut with a punch under sterile conditions from different fish skin preparation protocols (In this test, fresh fish skin without any process was used as internal control and epithelial cell culture on plate was used as external control) and placed in each well. After 24, 48, and 72 h of C619 culture, MTT (5 mg/mL; Sigma Chemical Co, USA) was added to each well of the monolayer cultures. The cultures were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. Finally, formazan salts were dissolved with 100 µL DMSO overnight, and the optical density was measured at 570 nm, using a microplate reader (Rayto Life and Analytical Sciences Co., Shenzhen, China).

2.2. Histological staining

2.2.1. Hematoxylin and Eosin (H&E)

Skin samples were cut into small pieces and fixed in 10 % formalin solution for 24 h, and embedded and blocked in paraffin wax. The implanted specimens were then cut perpendicular to the skin surface to obtain 5 µm-thick skin cross-sections. Each section was stained with hematoxylin and eosin and observed under a light microscope to study morphology and decellularized tissues (Tosta et al., 2017). Areas stained blue or purple indicate DNA, and areas stained pink indicate proteins.

2.2.2. Masson's trichrome

Masson's trichrome staining method is used to identify collagen fibers in tissues such as skin, heart, etc (Suvik and Effendy, 2012). To perform this type of staining, like H&E staining, skin samples were cut into small pieces and fixed in 10 % formalin solution for 24 h, and embedded and blocked in paraffin wax. The implanted specimens were then cut perpendicular to the skin surface to obtain 5 µm thick skin sections. Each section was stained and observed under a light microscope. In trichrome staining, the collagen fibers are blue and the cell nucleus is dark blue (purple) on a red background.

Table 1
Decellularization protocols of fish skin.

Protocols	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6
1	Freezing and thawing	Hypertonic solution 24h	–	–	Hypotonic solution 24h	Freeze dry
2	Freezing and thawing	Hypertonic solution 24h	T/E* 0.25 % W/V 90 min	–	Hypotonic solution 24h	Freeze dry
3	Freezing and thawing	Hypertonic solution 24h	Triton X100 1 % W/V 90 min	–	Hypotonic solution 24h	Freeze dry
4	Freezing and thawing	Hypertonic solution 24h	Triton X100 0.5 % W/V 24h	–	Hypotonic solution 24h	Freeze dry
5	Freezing and thawing	Hypertonic solution 24h	Triton X100 0.25 % W/V 24h	–	Hypotonic solution 24h	Freeze dry
6	Freezing and thawing	Hypertonic solution 24h	T/E 0.25 % W/V 90 min	Triton X100 0.5 % W/V 24h	Hypotonic solution 24h	Freeze dry
7	Freezing and thawing	Hypertonic solution 24h	T/E 0.25 % W/V 90 min	Triton X100 0.25 % W/V 24h	Hypotonic solution 24h	Freeze dry

* T/E : Trypsin-EDTA (0.25 %), T : Trypsin.

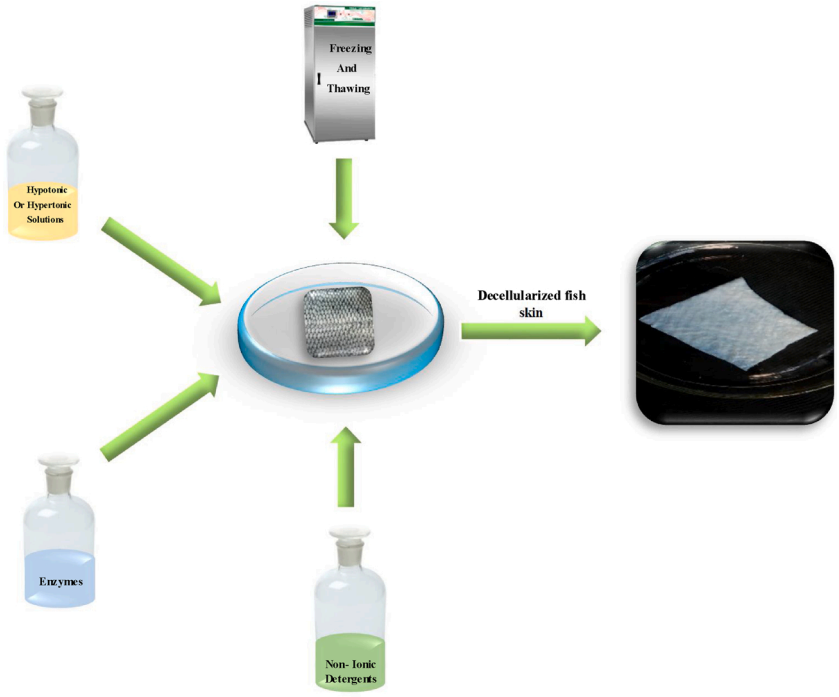

Fig. 1. Different protocols of the decellularization of fish skin samples.

2.3. Determination of DNA content

To evaluate the presence of DNA in the scaffold, 100 mg of the scaffold was weighed and pulverized in liquid nitrogen (Mohammadalipour et al., 2019). It was placed in a DNA-free microtube and the DNA was extracted according to the instructions of the manufacturer of the KIAZOL (Kiazist Life Sciences, Iran) DNA extraction kit. The amount of total DNA was measured by measuring the absorbance at 260 nm by a device (Eppendorf Photometer) and the amount of DNA per mg of tissue was calculated according to previous studies (Mohammadalipour et al., 2019).

2.4. Structural analysis

Changes in microstructure after cross-sectional decellularization were recorded by scanning electron microscopy (SEM) (Cambridge Stereo-scan (S-360)). To perform this imaging, the best decellularized samples with fresh fish skin were cut into small pieces and then covered with a thin layer of gold. The imaging process was performed by scanning electron microscopy at 20 V.

2.5. Physical & mechanical properties

2.5.1. Differential scanning calorimetry (DSC)

Denaturation temperatures of the ECM proteins after decellularization process were determined by Differential Scanning Calorimetry (DSC) analyze (Diamond DSC-Pekin Elmer) at temperature speed 0.5 °C/min and in the range of 20−70 °C.

2.5.2. Tensile strength

The mechanical properties of scaffolds were determined by uniaxial tensile testing. Fresh skin and decellularized tissue skin with thickness 0.25 mm were tapped with tissue paper to remove excess water, cut into 42 mm × 5 mm strips, and secured onto the tensile tester (Instron 5543, Instron, USA) with clamps in a 100 N load cell. The dimensions of the sample were measured using digital calipers and recorded in the tensile testing software. The load cell was calibrated, and the sample was pulled with an extension rate of 10 mm/min until the sample broke.

2.5.3. Degradability test

In this study, two methods were performed to investigate the degradability of biological scaffolds. In the first method, the freeze-dried skin was cut into 1 cm × 1 cm samples and its exact weight was measured and placed in 1 mL PBS on a shaker at 37 °C for 28 days. The samples were removed, washed, and dried with distilled water and measured their weights at different times. The amount of degradation of each sample was calculated in terms of weight loss according to the following formula (Eq. 1) (Lau et al., 2019).

$$\text{Weight loss } \% = \frac{W_i - W_f}{W_i} \times 100 \quad (1)$$

Where W_i represented the initial dry weight of skin (g) and W_f represented the dry weight of the recovery fragment of skin after the degradation (g). The percentage of weight loss was taken from the average of three sample.

In the second method, each 1 cm × 1 cm freeze-dried sample was accurately weighed and placed in 200 U/mL type I collagenase for enzymatic degradation. The tubes were exposed to 37 °C for 8 days. After each day, the weight of the sample was measured. Weight loss percentage was calculated according to the above formula.

2.6. Cell study

To evaluate the rate of cell proliferation, adhesion, and also cell morphology on the scaffolds, epithelial cells were cultured. To perform this process, decellularized scaffolds were cut into 1 × 1 cm^2 and implanted in 24 cell culture plates with 10,000 cells. Cells were cultured in whole cell culture medium with 10 % bovine fetal serum and penicillin / streptomycin and L-glutamine at 37 °C with 5 % CO_2. The culture medium was changed on average every day. The samples were dehydrated with alcohol and then stored with osmium tetroxide vapor at 4 °C for 2 h. The samples were kept in a dryer and dried, then covered with gold and examined by Scanning Electron Microscopy (SEM) (Cambridge Stereo-scan, S-360) analyze.

All data are expressed as mean ± standard error of the mean unless noted. Statistical analysis was performed using one way analysis of

variance (ANOVA) test. Differences between the means were considered statistically significant when $p < 0.05$.

3. Results and discussion

For selection of the best decellularization protocol, first was done toxicity test. According to the results of the MTT test (Fig. 2), the survival rate of epithelial cells on the decellularized scaffolds by protocol 4 is higher than other protocols even after 72 h. The results mean that decellularized skins by protocols 4 and 5 have less toxicity than other samples and also fresh fish skin (Internal control). The control sample and treated samples with protocol 1 were not showed a significant difference. The presence of cells within the extra cellular matrix (Internal control and Protocol 1) and also using detergents with different steps (Protocols 6 and 7) and effect of time parameter (protocols 2 and 3) can affect the toxicity. In addition, Protocols 4 and 5 with triton detergent showed less toxicity and better decellularization.

Fig. 3 shows histological staining of the decellularized skin samples by different methods. The fish fresh skin samples showed arrangement and density of the extracellular matrix and collagen network of the skin tissue. Structural order and morphological arrangement were maintained well with protocol 1, but the method couldn't completely separate and remove cells from matrix. According to previous study (Greco et al., 2015) that was used such physical method on pig skin, the results were completely different from the results of our study. They showed that completely cells removed from the pig skin thick tissue, while in our study, cells remained in the fish skin tissue (protocol 1). The decellularized sample obtained from protocol 4 had the highest efficiency in terms of network order and cell removal. This is due to the use of non-ionic detergents with the process of physical decellularization. Because the non-ionic detergents does not interact with the structural proteins of the extracellular matrix and only cause the cells to separate from the matrix. This result is consistent with the results of previous study (Farrokhi et al., 2018) that had been performed on rat skin that resulted in cell removal and network order. For samples obtained from protocols 6 and 7, with a combination of enzymatic, chemical, and physical methods, although the cells were removed from the tissue, but the structural order of the extracellular matrix was lost.

Table 2 shows the amounts of residual DNA content in decellularized tissues. It was 415.5 ± 4.6 ng in the skin tissue per mg of fish skin (Control). After decellularization processes, a significant reduction in the amount of DNA content illustrated in the tissues compared to the control sample. The highest amount of DNA in the tissue was related to the decellularized sample by protocol 1. This amount of residual DNA content in the tissue is quite different from the amount of DNA reported in previous studies on pig skin (Greco et al., 2015). The result of the amount of DNA content in the tissue was compared to the conducted study by Farrokhi (Farrokhi et al., 2018). They reported amount of DNA content almost zero nanograms per milligram of tissue after the decellularization process on rat skin by the non-ionic detergent Triton X100 (0.5 % v/v). Lau (Lau et al., 2019) and their colleagues obtained the amount of DNA content remaining in the tilapia skin tissue about 1.8 ng / mg. However, according to other study (Kwon and Moon, 2018), the amount of cells remaining in the tissue was not completely removed from the tissue, but the amount of DNA content remaining in the tissue reached less than 50 ng/mL, that cannot cause immune stimulation processes and inflammation (Kwon and Moon, 2018). According to the results of our study, all decellularization methods except the physical process were able to remove the cells as much as allowed, of which the decellularized samples from protocol 4 had the lowest amount of cell contents. In addition, the samples from protocol 4, due to the use of a combination of physical and chemical methods and having low toxicity, less DNA, and keeping morphological arrangement & integration showed better results and selected for other analyses.

Fig. 4A shows the result of temperature analysis by DSC technique. The first peak is a positive peak, which indicates the glass transition temperature (T_g) of decellularized skin sample based on protocol 4. This temperature is the beginning of the change in the properties of the polymers to initiate the mobility between the polymer chains. The structural proteins of the extracellular matrix showed a temperature of 24–68 °C and also, maximum peak of the temperature changes at 54.4 °C. Denaturation temperature of human skin has been reported about 54.8 °C by wiegand and their colleagues (Wiegand et al., 2013). So, our decellularized skin sample was similar to human skin in terms of temperature changes. It was shown that the denaturation temperature of each type of collagen is different in different parts of the body (Not et al., 2013). On the other hand, the density and the presence of other materials in the sample under analysis can also change this temperature. In addition, the denaturation temperature of decellularized skin and human skin showed same similarities. On the other hand, a scaffold that be placed in the body must withstand body temperature and the temperature of inflammation after transplantation, which is about 42 °C. So, the decellularized fish skin will be able to withstand body temperature and inflammation.

Fig. 4B shows tensile strength results of the fresh fish skin and scaffolds obtained from the decellularization process. To perform this test, the decellularized skin by protocol 4 and fish skin (Control) samples were applied and results were compared with each other. The maximum tensile stress and Young modulus for the fresh fish skin sample with a thickness of 0.12 mm were 32.77 MPa and 284.14 MPa, respectively.

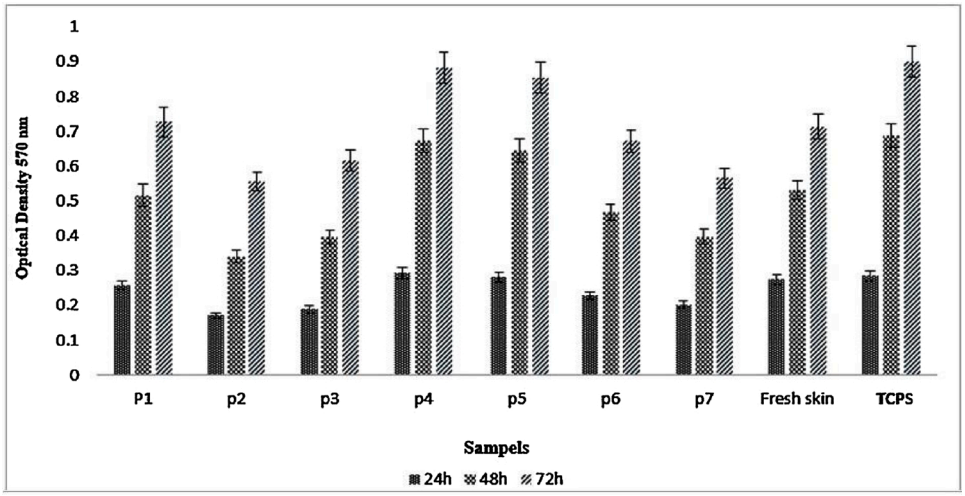

Fig. 2. Viability of epithelial cells on samples with different protocols and external control (TCPS).

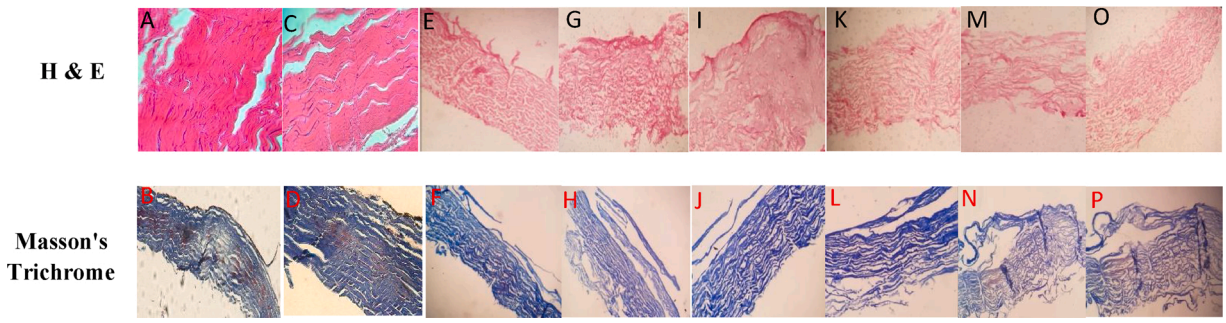

Fig. 3. Staining images (Cross-sectional view) of the decellularized samples by different protocols. Fresh skin as control(A and B); Protocol 1(C and D); Protocol 2(E and F);Protocol 3(G and H);Protocol 4(I and J);Protocol 5(K and L);Protocol 6(M and N);Protocol 7(O and P).Bar =50 μm.

Table 2
DNA contains of the decellularized skin samples by different protocols.

	Samples							
	Control	1	2	3	4	5	6	7
DNA (ng/mg)	415.5 ± 4.6	346.2 ± 2.2	30.5 ± 3.8	29.8 ± 1.8	20.5 ± 1.2	28.1 ± 2.9	27.8 ± 3.8	25.7 ± 2.1

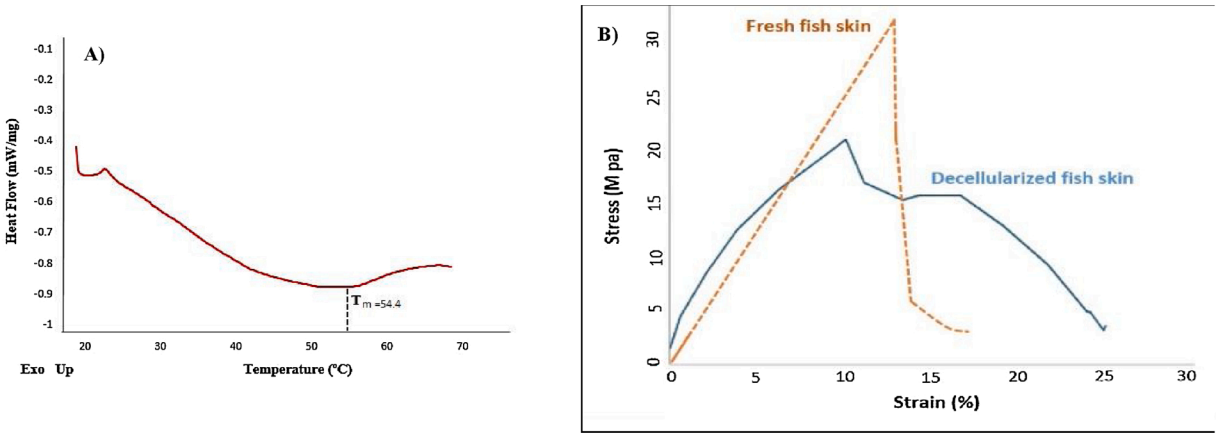

Fig. 4. DSC diagram of the decellularized skin by protocol 4 (A), mechanical test of the fish fresh skin, and sample obtained by the decellularization process (B).

While the decellularized skin sample showed tensile stress and Young modulus about 21.25 MPa and 192.15 MPa, respectively.

In a study (Brouki Milan et al., 2020) on human skin, the results of tensile strength showed that healthy human skin has Yang modulus about 245.4 ± 12 MPa and after the decellularization process decreased to 196 MPa. The tensile strength results of tilapia skin sample with a thick showed (Lau et al., 2019) the maximum tensile stress 35 MPa and Young's modulus about 143 MPa that after decellularization process reduced to 56 and 24 MPa, respectively. These results indicate that despite the thin skin of fish, the extracellular matrix network after decellularization largely maintains its structure and entanglement and is able to withstand tensile forces.

Fig. 5 shows degradability rate of the modified scaffolds by the decellularization process with 2 different methods. In the first method, the decellularized skin samples were placed in a phosphate buffer solution (PBS) at 37 °C in a shaker for 28 days. During the specified time point, the samples were weighed and the degradation percentage was calculated in terms of weight loss percentage. Results showed that the decellularized skin samples were destroyed by 50 % after 28 days in phosphate buffer solution. In the second method, we exposed the decellularized skin sample to collagenase enzyme and the samples were weighed during certain times and calculated the percentage of scaffold destruction in terms of weight loss percentage. Results showed that the decellularized skin samples were completely degraded after 8 days. The previous studies (Lau et al., 2019; Kwon and Moon, 2018) showed that tilapia fish skin has degradability rate about 81 % in PBS solution, and complete degradation in enzymatic media after 3 days.

Fig. 6B shows images from the scanning electron microscopy of the decellularized sample, compared to the fresh fish skin (6A), in cross section, to observed changes in the structure of the skin after the decellularization process. Fig. 6C and D showed cell adhesion on the re-cellularized samples by SEM at different magnifications. Images showed that epithelial cells have good adhesion on the decellularized matrix.

4. Conclusion

Fish skin was decellularized by various physical, chemical and enzymatic methods to create a suitable alternative as a scaffold instead of synthetic materials to regenerate skin tissue. Using a combination of physical and chemical methods with the help of non-ionic detergent, cells were separated well from the extracellular matrix, and samples preserve structural arrangement and order. The decellularized skin showed high biocompatibility and low toxicity with suitable physical & mechanical properties and also good cell adhesion for human skin tissue regeneration. The best sample in terms of structure, toxicity, cell adhesion, and mechanical & physical was related to the decellularized skin (Protocol 4) by combinations of hypertonic & hypotonic solutions and Triton X100 (0.5 %). In addition, the used protocol in this study can

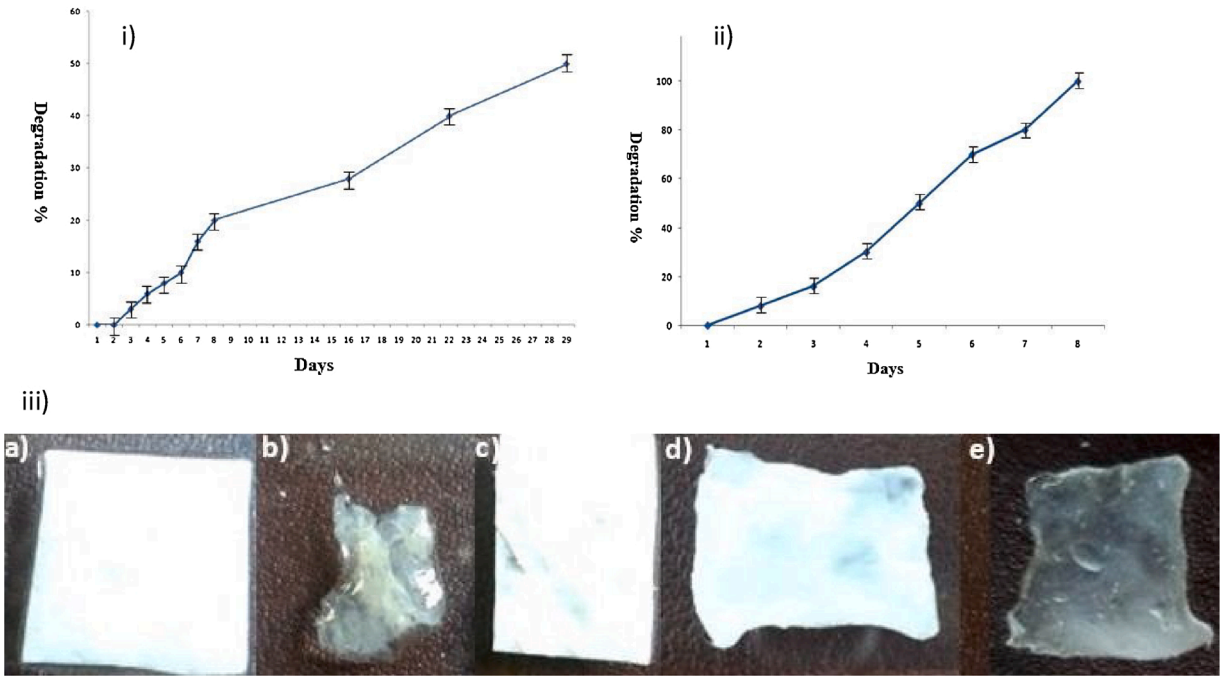

Fig. 5. Degradation diagrams of the decellularized skin sample in PBS (i), and collagenase enzyme (ii).Degradation morphology of the decellularized skin in PBS after 28 days (iii). Decellularized fish skin (a); degradation in collagenase enzyme after 5 days (b); degradation in PBS after 7 (c), 14 (d), and 28 days (e).

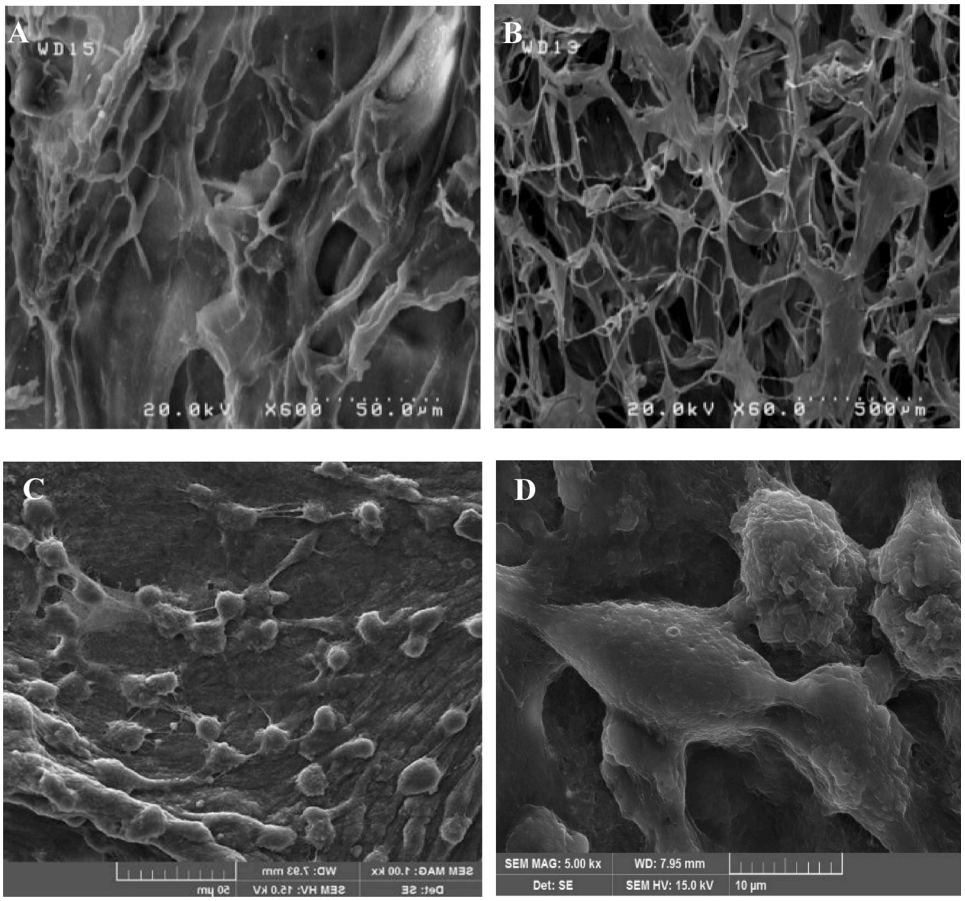

Fig. 6. SEM images of the fish fresh skin (A), decellularized skin (B), and re-cellularized skin at different magnifications (C and D).

be a suitable method for design of acellular matrixes in tissue regeneration.

Declaration of Competing Interest

The authors report no declarations of interest.

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