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Decellularized human amniotic membrane: more is needed for an efficient dressing for protection of burns against antibiotic-resistant bacteria isolated from burn patients

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ABSTRACT

Human amniotic membranes (HAMs) have attracted the attention of burn surgeons for decades due to favorable properties such as their antibacterial activity and promising support of cell proliferation. On the other hand, as a major implication in the health of burn patients, the prevalence of bacteria resistant to multiple antibiotics is increasing due to overuse of antibiotics. The aim of this study was to investigate whether HAMs (both fresh and acellular) are an effective antibacterial agent against antibiotic-resistant bacteria isolated from burn patients. Therefore, a HAM was decellularized and tested for its antibacterial activity. Decellularization of the tissue was confirmed by hematoxylin and eosin (H&E) and 4,6-diamidino-2-phenylindole (DAPI) staining. In addition, the cyto-biocompatibility of the acellular HAM was proven by the cell viability test (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide, MTT) and scanning electron microscopy (SEM). The resistant bacteria were isolated from burns, identified, and tested for their susceptibility to antibiotics using both the antibiogram and polymerase chain reaction (PCR) techniques. Among the isolated bacteria, three bla_{IMP} gene-positive *Pseudomonas aeruginosa* strains were chosen for their high resistance to the tested antibiotics. The antibacterial activity of the HAM was also tested for *Klebsiella pneumoniae* (American Type Culture Collection (ATCC)

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700603) as a resistant ATCC bacterium; *Staphylococcus aureus* (mecA positive); and three standard strains of ATCC bacteria including *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27833), and *S. aureus* (ATCC 25923). Antibacterial assay revealed that only the latter three bacteria were susceptible to the HAM. All the data obtained from this study suggest that an alternative strategy is required to complement HAM grafting in order to fully protect burns from nosocomial infections.

1. Introduction

Human amniotic membrane (HAM) is a multilayer membrane consisting of epithelial cells, fibroblasts, and basement membrane [1]. Some of its favorable characteristics make HAM a promising scaffold for tissue engineering applications. Functioning as an extracellular matrix (ECM), the basement membrane of HAM is rich in hyaluronic acid [2], collagen types I, III, IV, V and VI, lamini, elastin, fibronectin, proteoglycans, etc. [1,3]. The presence of such components in the ECM endows some unique properties to HAM. For example, HAM promotes cell proliferation and maturation. In addition to its easy availability and cost-effectiveness, some of the potential benefits of HAM in tissue engineering applications include its low immunogenicity and high antibacterial property [4,5]. The application of HAM in skin tissue engineering has been suggested for many years now [5]. This ideal scaffold has been used widely in skin dressing [6,7], neurosurgery [8], and ophthalmic [9] and vaginal surgeries [10] in its fresh or decellularized forms. Decellularization of the HAM has been reported to enhance its cell proliferation-supporting function and reduce its immunogenicity [3,11].

Many studies have reported the antibacterial property of the HAM [1,4,5]. In addition to its antibacterial activity, HAM promotes the healing of infected wounds [4]. On the other hand, the prevalence of antibiotic-resistant bacteria is increasing due to the overuse of antibiotics, especially in developing countries. Development of multidrug-resistant (MDR) strains has become a major concern in the health-care community [12–14]. The aim of this study was to investigate whether HAM (both fresh and decellularized) can protect burns from antibiotic-resistant infections, as an ideal burn wound dressing.

2. Materials and methods

2.1. Preparation of decellularized HAM

2.1.1. Tissue collection

The placentas were collected, and the HAM was separated by a procedure described in our previous work [15]. Briefly, the tissues were obtained from consenting mothers at the time of their cesarean section deliveries. The samples were screened serologically for the possibility of human hepatitis virus types B and C, syphilis, human immunodeficiency virus types I and II, gonorrhea, cytomegalovirus, and toxoplasmosis. The tissues were embedded in an antibiotic-supplemented phosphate-buffered saline (PBS). The human placenta collection was carried out in accordance with the Declaration of Helsinki [16].

The fresh placentas were rinsed with sterile distilled water. After removal of residual blood from the tissue, the amniotic membrane was separated from the chorion and used for the following decellularization process. All steps were carried out under sterile conditions.

2.1.2. Decellularization

For the purpose of decellularization, the fresh HAM was treated with 0.5 M NaOH and 0.2% ethylenediaminetetraacetic acid (EDTA) for 30 s and 30 min, respectively. The tissue was then embedded in 5% ammonium chloride and shaken vigorously, followed by scraping with a cell culture scraper. Both the decellularized and fresh samples were washed with sterile PBS, maintained at −80 °C, and then freeze-dried under vacuum. The tissues were sealed with nylon and stored in room temperature until the following experiments.

2.1.3. Characterizations

The tissue was fixed with 10% natural-buffered formalin (Sigma), followed by dehydration through a graduated series of increasing ethanol and embedment in paraffin. The samples were sectioned with thicknesses of 4 μm. The fresh HAM (Fig. 1A, C, and D) and acellular (Fig. 1B, E and F) tissues were stained with hematoxylin and eosin (H&E) (Fig. 1A and B) and 4′,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich, USA; Fig. 1C–F), and then they were viewed under light and ultraviolet microscopes, respectively, to confirm decellularization of the tissue. The results confirmed that the cells were successfully removed from the tissue after the decellularization process (Fig. 1).

2.2. Isolation and identification of resistant bacteria

2.2.1. Isolation and identification of bacteria from burn wounds

The bacteria were collected from the wound exudate of patients with burns admitted to the Burn Unit at the Shahid Motahari Hospital (Tehran, Iran). The bacteria were then identified using standard methods such as ‘‘oxidase,’’ ‘‘triple sugar iron,’’ ‘‘oxidative–fermentative,’’ and ‘‘motility’’ tests [17].

2.2.2. Polymerase chain reaction

The polymerase chain reaction (PCR) technique was used for screening of the β-lactamase imipenemase (bla_{IMP}) gene, encoding a protein conferring widespread resistance in

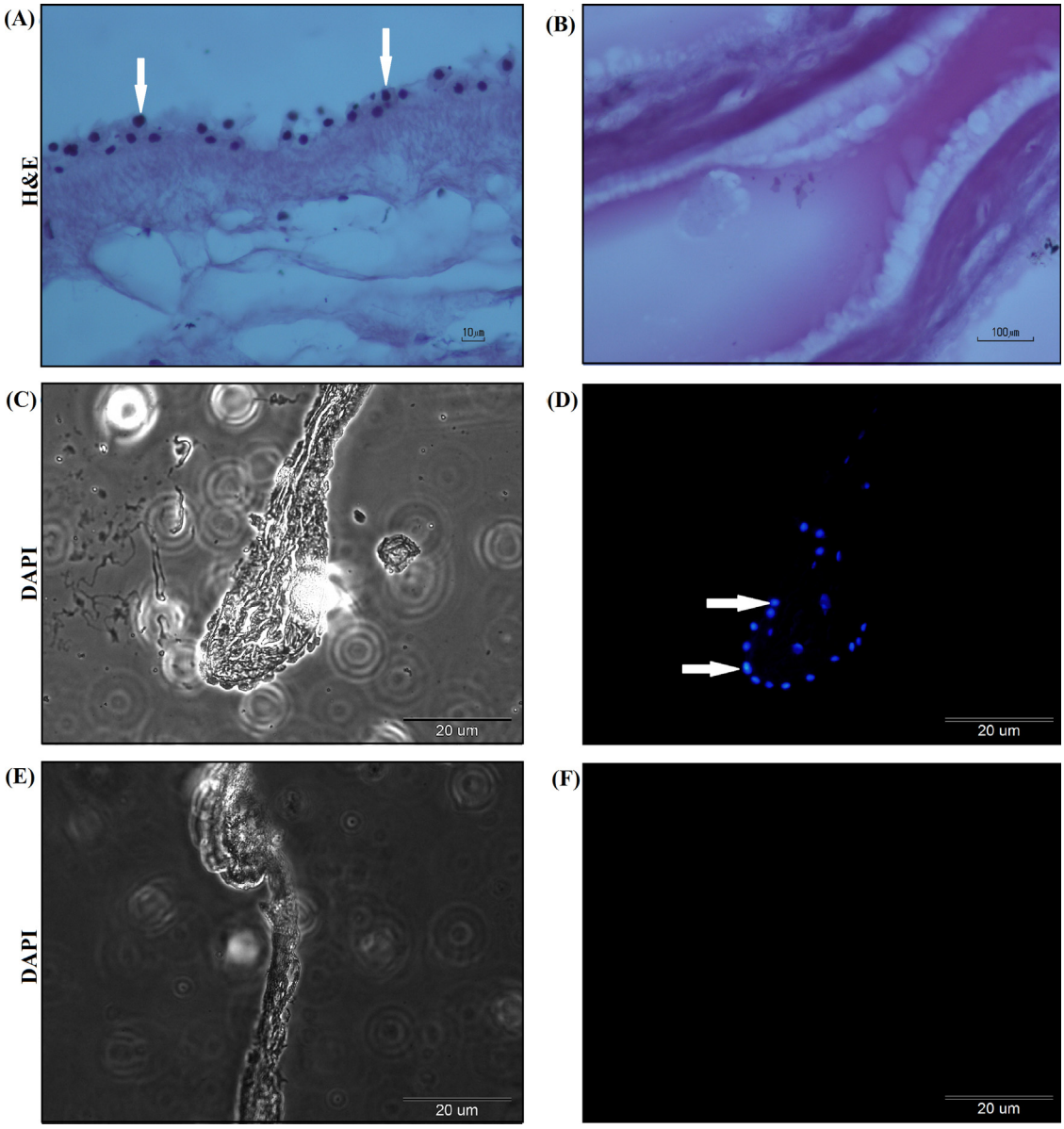

Fig. 1 – H&E-stained samples of fresh (A) and acellular (B) HAMs under light microscope. DAPI-stained fresh HAM under light (inverted microscope system) (C) and ultraviolet (D) microscopes. DAPI-stained acellular HAM under light (inverted microscope system) (E) and ultraviolet (F) microscopes. White arrows indicate the cells within the HAM.

bacteria [18]. For this purpose, the total DNA content of the different bacterial isolates was extracted using the DNA extraction kit (Bioneer Company, Daejeon, Korea, Cat. number K-3032-2). PCR was used for screening of the bla_{IMP} gene under conditions reported previously [19]. The sequences of the primers used for amplification of the bla_{IMP} gene were as follows: IMP-forward (5′-GAAGGCGTTTATGTTCATAC-3′) and IMP-reverse (5′-GTAAGTTTCAAGAGTGATGC-3′). The PCR mixture contained the DNA template, forward/reverse primers, and the master mix (Bioneer Company, Daejeon, Korea, Cat. number K-2016). The PCR purification kit (Bioneer Co., Daejeon, Korea) was used to purify the PCR products, and sequencing was performed by the Bioneer Company (Daejeon, Korea). The nucleotide sequences were then analyzed using the Chromas 1.45 software and the BLAST program from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST).

2.2.3. *Antimicrobial susceptibility testing*

The susceptibility of bla_{IMP}-positive bacteria to commonly used antibiotics was investigated using the Kirby–Bauer disk diffusion test according to Clinical Laboratory Standards Institute (CLSI) 2013 guidelines [20]. The following antibiotic disks were used in this study, all of which were purchased from MAST Co. (Mast Diagnostics, UK): tobramycin (TOB, 10 μg), piperacillin/tazobactam (PTZ, 100/10 μg), ceftriaxone (30 μg), piperacillin (100 μg), meropenem (10 μg), ceftazidime (30 μg), carbenicillin (Car,100 μg), amikacin (AK, 30 μg), imipenem (10 μg), cefepime (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), and aztreonam (30 μg).

2.3. Susceptibility of bacteria to HAM samples

Among the isolated bacteria, three resistant *Pseudomonas aeruginosa* strains (bla_{IMP} positive) were chosen and tested for their susceptibility to HAM samples. In addition, *Klebsiella pneumoniae* (American Type Culture Collection (ATCC) 700603), as a resistant ATCC bacterium; *Staphylococcus aureus* (mecA positive); and three standard strains of ATCC bacteria including *Escherichia coli* (ATCC 25922), *P. aeruginosa* (ATCC 27833), and *S. aureus* (ATCC 25923) were used in this study. The susceptibility of the abovementioned bacteria to the HAM samples (both fresh and acellular) was tested using the Kirby–Bauer disk diffusion test, as described earlier. For this purpose, the bacteria were cultured in a Mueller-Hinton agar (MHA) plate. The samples were stretched on aluminum sheets, placed in the middle of the MHA agar plate, and then incubated for 24 h at 37 °C. After incubation, the percentage of plates with an inhibition zone as well as the diameter of the inhibition zone around each specimen was measured. The inhibition zone was defined by the following formula (1):

$$\text{Inhibition zone (mm)} : \text{diameter of inhibition zone} - \text{diameter of HAM on agar plate} \quad (1)$$

2.4. Cellular response to the acellular HAM

2.4.1. Cell culture

NIH 3T3 fibroblast cells were used for evaluation of the in vitro cellular response to the HAM. For this purpose, the cells were purchased from the Pasteur Institute of Iran and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% nonessential amino acids (all from Gibco, Carlsbad, CA, USA). The cells were seeded on both fresh and acellular tissues, and they were incubated at 37 °C in a 95% humidified atmosphere of 5% CO_2 for 72 h. After the prescribed duration of incubation, the cell viability and cell adhesion were evaluated by the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) test and scanning electron microscopy (SEM), respectively.

2.4.2. Cell viability

The cell viability test was conducted with a procedure described in our previously published works [21,22]. Briefly, the culture medium was replaced with fresh DMEM containing 10% MTT solution, and it was left in a cell culture incubator for 2 h. Then, the MTT solution was removed, and the samples were treated with dimethyl sulfoxide (DMSO) for 20 min. The viability of the cells was quantified by measuring the optical density (OD) of the samples using an ELISA (enzyme-linked immunosorbent assay) reader at a wavelength of 590 nm with a reference filter of 620 nm. The cells cultured in medium without HAM served as the control.

2.4.3. Cyto-biocompatibility and adhesion

The morphology of the cells cultured on the HAM samples was observed by SEM (Philips XL30, Eindhoven, the Netherlands) in order to evaluate the cell adhesion property of the samples. For this purpose, the samples were prepared for obtaining SEM micrographs by a procedure described in our previously published articles [23,24]. In brief, the cell-HAM samples were fixed with 2.5% glutaraldehyde (GA) solution and dehydrated through a graduated series of increasing acetone (Merck, Kenilworth, NJ, USA) up to 100%. The samples were then treated with 0.1% osmium tetroxide (OsO_4) (Sigma, USA), washed twice with PBS, and then dried through sublimation. The samples were sputter-coated with gold and observed by SEM at an acceleration voltage of 15 kV.

2.5. Statistical analysis

The Kolmogorov–Smirnov test was used for assessing the distribution of the data. The data were statistically analyzed by independent sample *t*-tests and one-way analysis of variance (ANOVA) where appropriate, and they were expressed as a means of ±standard deviation (SD). All of the data obtained from the MTT assay were normalized to a positive control (as 100% cell viability). A p-value of <0.05 was considered as the level of significance. The data were collected, and the SigmaPlot 11.0 θ software was used for plotting graphs.

3. Results and discussion

As a membrane that surrounds and protects the fetus, HAM comprises three main layers containing cells and ECM. HAM profoundly affects cell proliferation and function [1,9,25]. In addition, the presence of ECM proteins in HAM such as cytokines and growth factor makes it a promising antibacterial agent [3,26]. The abovementioned properties as well as its inexpensiveness and easy availability make HAM a potential candidate for surgical interventions. On the other hand, decellularization of HAM not only helps reduce the possibility of graft rejection but also enhances its cell proliferation-supporting function [3,27]. The antibacterial property of HAM was reported by many studies [5,26,28]. However, the antibacterial activity of such a scaffold against resistant bacteria isolated from burn patients is controversial. A major concern in burn centers, the overuse of antibiotic therapy leads to rapid increase of resistant bacterial strains [12,13]. Therefore, the isolation and identification of antibiotic-resistant bacteria and the development of a favorable wound dressing with high antibacterial activity are very important issues. The aims of the current study were not only to investigate the effect of the decellularization process on the antibacterial property of the HAM, but also to evaluate the effectiveness of such a membrane in the prevention and treatment of MDR bacteria isolated from burn patients. Herein, the bacteria were isolated from burns and identified, and their resistance to commonly used antibiotics was assessed by both PCR and antibiogram test. The HAM was then decellularized and stored at room temperature. After characterization of the decellularization process, the antibacterial activity of both the fresh and acellular tissues was tested using the Kirby–Bauer disk diffusion method on MHA. Furthermore, the cytotoxicity and cell adhesion properties of both tissues, as two critical parameters in tissue engineering scaffolds, were tested. The methodology followed in this study is illustrated in Fig. 2.

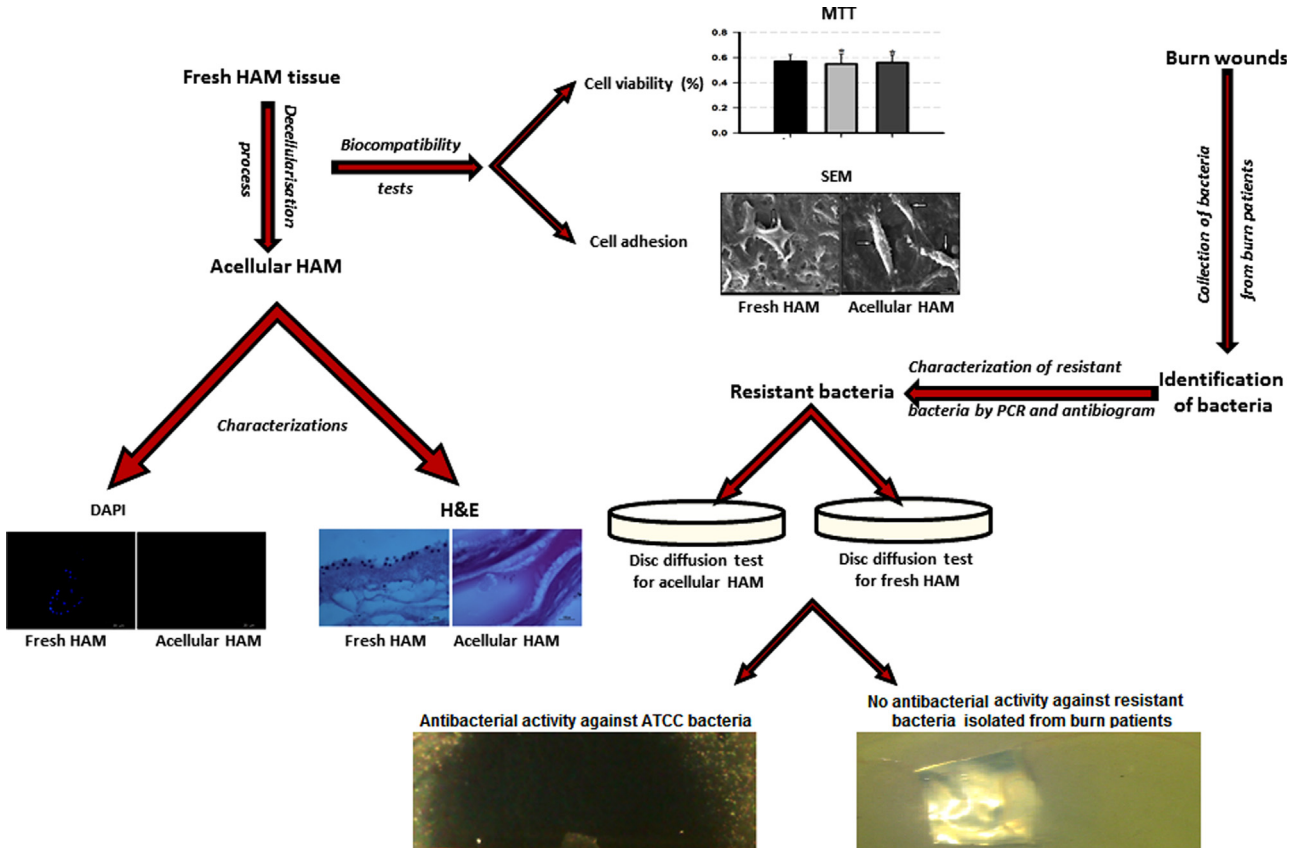

Fig. 2 – The methodology followed in this study.

3.1. *PCR for bla_{IMP} gene*

PCR was performed to screen the bla_{IMP} gene-positive bacteria. PCR and sequencing methods revealed that some isolates were positive for the bla_{IMP} gene. The results showed a conserved region for the restriction sequence of bla_{IMP}-1 gene, which was confirmed by BLAST in NCBI. The nucleotide sequence data reported in this paper have been submitted to the GenBank sequence database (accession no. JX648311 and JX644173). The bla_{IMP} gene, encoding metallo-β-lactamase, is one of the most common genes isolated from resistant bacteria. The bla_{IMP} gene cassette is disseminated into various gram-negative bacterial species through class I integrons, located on resident plasmids or bacterial genome, thus leading to antibiotic resistance. Among the bacteria isolated from burn patients, three highly resistant bacteria (IMP-producing *Pseudomonas* strains) were chosen in this study.

3.2. *Antimicrobial susceptibility testing*

All IMP-producing *P. aeruginosa* strains were found to be resistant to aztreonam, TOB, ciprofloxacin, PTZ, cefepime, ceftriaxone, carbenicillin, meropenem, imipenem, ceftazidime, gentamicin, and AK. The results indicated that 100% of bla_{IMP}-positive isolates were MDR.

3.3. *Susceptibility to HAM samples*

To date, many antibacterial agents have been developed for prevention of infections by evaluating their antibacterial activity against standard strains of bacteria [29,30]. The challenges in the prevention and treatment of infections in injuries, especially burns, are associated with the presence of MDR bacteria. Therefore, the antibacterial activity of such agents against resistant bacteria is critical. To address these challenges, the antibacterial activity of both fresh and decellularized HAM was assessed against both standard strain of bacteria such as *E. coli*, *S. aureus*, and *P. aeruginosa* (which are not resistant to commonly used antibiotics) and other antibiotic-resistant bacteria. Two resistant standard strains of *K. pneumoniae* (ATCC 700603) and *S. aureus* (mecA positive) and three resistant *P. aeruginosa* strains isolated from burn patients (IMP-producing *P. aeruginosa*) were tested for their resistance to HAM. Figs. 3–5 show a growth inhibition zone around the HAM samples after 24 h of incubation at 37 °C. According to our results, both fresh and acellular HAM showed a strong antibacterial activity against *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), and *P. aeruginosa* (ATCC 27853) (Fig. 3). However, the inhibitory effects were not observed against the other tested bacteria (Figs. 4 and 5). The results of the mean value and maximum size of the inhibition zone around the tissues is shown in Table 1.

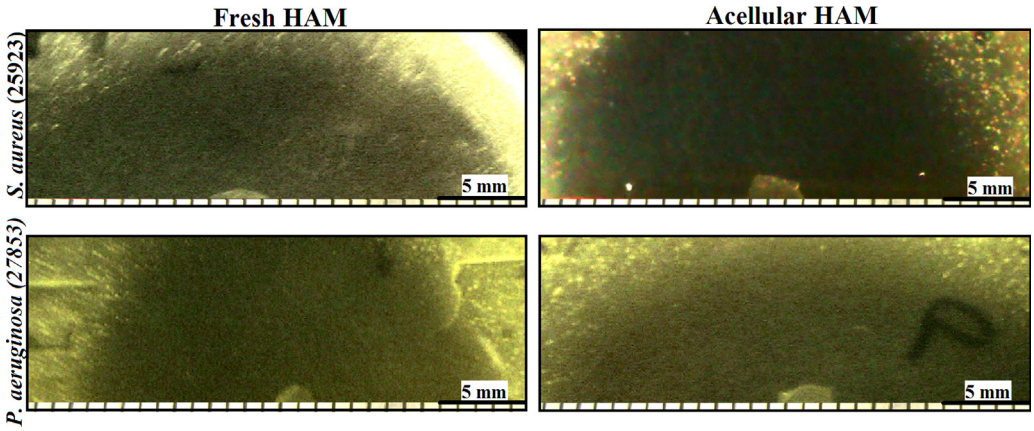

Fig. 3 – Disk diffusion test. A growth inhibition halo was observed around the HAM tissues (fresh and acellular) for *S. aureus* (ATCC 25923) and *P. aeruginosa* (ATCC 27853).

According to our observations, although the HAM samples did not inhibit the growth of resistant *P. aeruginosa* isolated from burn patients, it was found to be a powerful pigmentation inhibitor in these bacteria (Fig. 4). *P. aeruginosa* produces two types of pigments, a bluish green pigment (called also pyocyanin) and a fluorescent yellow-green pigment (called also pyoverdin). Both pyocyanin and pyoverdin have been found to be important virulence factors. Pyocyanin acts as a virulence factor because of its ability to produce superoxide radicals and hydrogen peroxide through the reduction of oxygen. Pyoverdin, however, does not act directly as a virulence factor. This pigment is considered as a survival factor that enables the microorganism to survive as a pathogen [31–34]. Nevertheless, our observations need further molecular investigations to clarify the effects of HAM on the expression of genes that confer virulence to these bacteria. Taken together, our data indicated the low efficiency of the HAM tissues in preventing the growth of the resistant bacteria.

Nosocomial infections continue to be a common, difficult, and controversial challenge faced in clinical practice, especially in burn patients. It has been reported that deep partial burn wounds require a tissue engineering dressing to not only promote wound healing but also prevent infections [35]. Many wound dressings have been introduced for their efficiency in the treatment of burns. Among them, HAM has many promising features that make it an ideal scaffold for wound healing [36–40]. For example, Mohammadi et al. [40] grafted fresh HAM in patients with chronic infected burn wounds. They showed that HAM interestingly increased the rate of graft take and exhibited promising antibacterial activity.

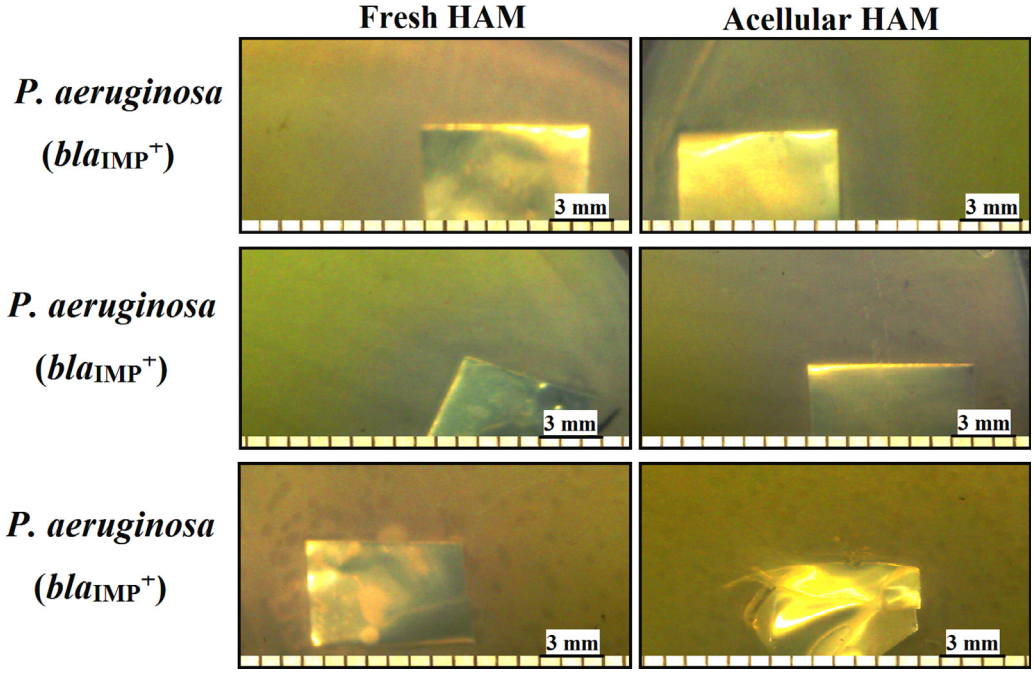

Fig. 4 – Disk diffusion test. No growth inhibition halo was observed around the HAM tissues (fresh and acellular) for three bla_{IMP}^{+} *P. aeruginosa* strains isolated from burn patients. The inhibition of pigmentation is clearly seen in this figure.

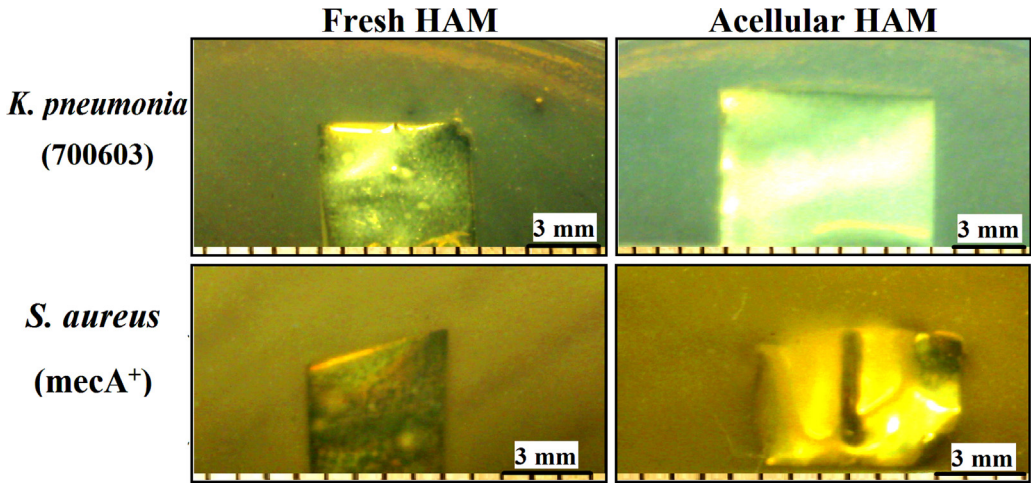

Fig. 5 – Disk diffusion test. No growth inhibition halo was observed around the HAM tissues (fresh and acellular) for two standard strains of *K. pneumoniae* (ATCC 700603) and *S. aureus* (mecA positive).

However, the abuse of antibiotic therapy contributes to the prevalence of antibiotic-resistant bacteria [41]. Therefore, development of an ideal burn wound dressing with efficient healing and antibacterial property is required for full treatment of burns. The results obtained from this study indicated that HAM may not be able to fully protect burns against the resistant bacteria, despite showing a strong antibacterial activity against the ATCC standard bacteria. Therefore, an alternative strategy, such as cross-linking the HAM with a powerful antibacterial agent, may tackle this hurdle. Therefore, researchers have focused on the development of a novel antibacterial agent [42,43]. For example, many studies have reported the widespread antimicrobial property of silver and fluoride [44–46]. Even in low concentrations, these substances have a biocidal property by damaging the DNA and RNA molecules and inhibiting protein synthesis [47]. The toxicity property of these materials is concentration dependent [43]. Therefore, achieving an optimal concentration of such materials with high toxicity effect against bacteria is worth investigating. It is important to note that the antibacterial agent must be safe and free of side effects. In a study, our research group developed a silver-doped bioactive glass (Ag-BG) with different molar ratios of silver. Then, the antibacterial property of the synthesized Ag-BG was evaluated. In addition, the responses of the human cells and immune system to the Ag-BG were investigated in vitro and in vivo, respectively [43].

Table 1 – Antibacterial activity of fresh and acellular human amniotic membrane on the number of examined plates cultured with resistant and ATCC bacteria as well as the mean and maximum size of inhibition zone.

Bacterial strain	Fresh amniotic membrane			Acellular amniotic membrane		
	Plates with inhibition zone (%)	Mean inhibition zone[a] (mm)	Maximum inhibition zone[a] (mm)	Plates with inhibition zone (%)	Mean inhibition zone[a] (mm)	Maximum inhibition zone[a] (mm)
E. coli (ATCC 25922)	100 (10^b,10^c)	22	23	100 (10^b,10^c)	23	25
S. aureus (ATCC 25923)	100 (10^b,10^c)	26	28	100 (10^b,10^c)	24	25
P. aeruginosa (ATCC 27853)	100 (10^b,10^c)	25	26	100 (10^b,10^c)	22	23
K. pneumoniae (ATCC 700603)	0 (10^b,0^c)	0	0	0 (10^b,0^c)	0	0
S. aureus (mecA positive)	0 (10^b,0^c)	0	0	0 (10^b,0^c)	0	0
P. aeruginosa (bla_{IMP} positive)	0 (10^b,0^c)	0	0	0 (10^b,0^c)	0	0
P. aeruginosa (bla_{IMP} positive)	0 (10^b,0^c)	0	0	0 (10^b,0^c)	0	0
P. aeruginosa (bla_{IMP} positive)	0 (10^b,0^c)	0	0	0 (10^b,0^c)	0	0

[a] (Diameter of inhibition zone – diameter of amniotic membrane sample).
[b] The number of plates examined for elimination of bacterial growth.
[c] The number of plates with inhibition zone or elimination.

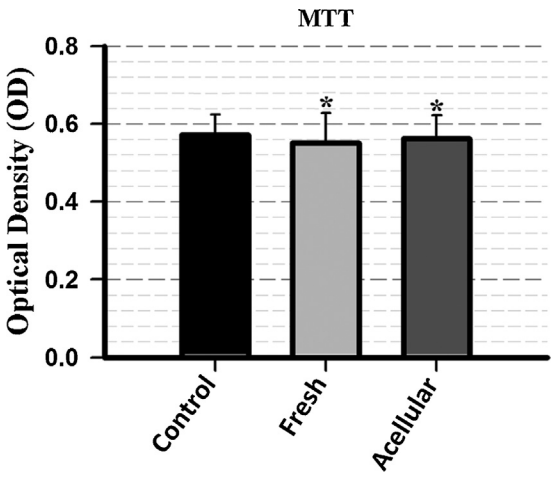

Fig. 6 – The MTT results of control (cell cultured in culture plate), fresh (cells cultured on fresh HAM), and acellular (cells cultured on acellular HAM) samples after 72 h of incubation at 37 °C in a 95% humidified atmosphere of 5% CO_2. The asterisk indicates no significant difference with control samples ($p > 0.05$, independent sample *t*-test).

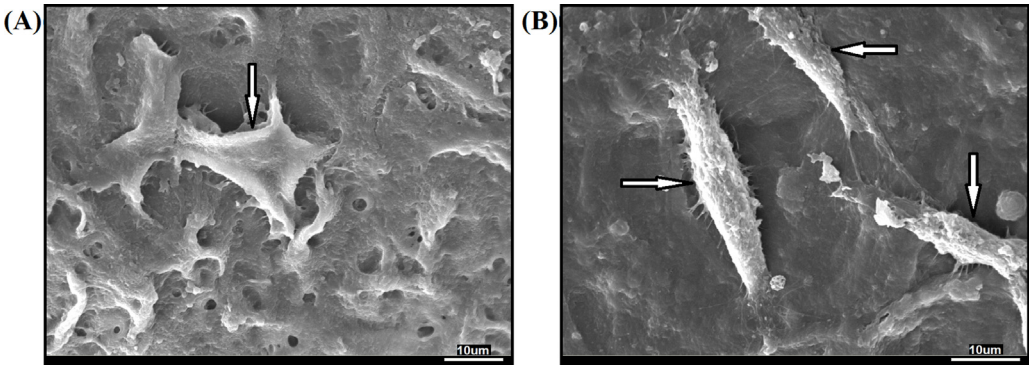

Fig. 7 – The SEM micrographs of the NIH 3T3 cells on fresh (A) and acellular (B) HAM samples. White arrows indicate the cells grown on the tissue.

Therefore, the aim of the future study would be to cross-link the acellular HAM with such efficient antibacterial substances.

3.4. *Cellular response*

The non-cytotoxicity property is one of the important features of tissue engineering scaffolds. Therefore, the evaluation of the cytotoxicity property after preparation and decellularization of the HAM samples is essential. To this end, the NIH 3T3 fibroblast cells were seeded on both fresh and acellular tissue and left at 37 °C for 72 h, as described earlier. Then, the cell viability and cell–tissue interaction were investigated by the MTT test and SEM, respectively, as described in the following sections.

3.4.1. *Cell viability*

The living cells are able to reduce tetrazolium salt (MTT) in their mitochondria. Therefore, the rate of MTT reduction indicates the level of cell viability [48]. The result obtained from the MTT test is shown in Fig. 6. As can be seen, there was no significant difference between the ODs obtained from fresh and acellular samples compared with the control, indicating the absence of the cytotoxicity effect of HAM ($p > 0.05$, independent sample *t*-test) (Fig. 6).

3.4.2. *Cell–tissue interaction*

The morphology of the cells cultured on the HAM samples was observed by SEM micrographs (Fig. 7). The cells were spread and attached actively to both fresh (Fig. 7A) and acellular (Fig. 7B) tissues, indicating favorable cyto-biocompatibility of the tissues before and after decellularization. These data confirmed the data obtained from the MTT test (Fig. 7).

4. Conclusion

Infections in burn wounds negatively affect the quality of wound healing. Survival of burn patients depends not only on the use of an efficient burn wound healing strategy but also on an effective strategy for preventing burn infections. HAM has been suggested as a favorable skin dressing of burn wounds due to its promising ability to heal burns and protect against bacterial infections. However, the increased prevalence of antibiotic-resistant bacteria in medical care centers is

considered a major challenge to the use of such dressing scaffolds. In this study, we found that among the bacteria isolates, some bacteria were highly resistant to multi-antibiotics that are commonly used in burn centers. Our data revealed that neither fresh nor acellular HAM was able to inhibit the growth of these bacteria in vitro. Therefore, an alternative strategy is required to complement HAM grafting in order to fully protect burn wounds against nosocomial infections.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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