The effects of preservation procedures on antibacterial property of amniotic membrane

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Amniotic membrane (AM), the innermost layer of the fetal membranes, has been widely employed in the surgical reconstruction and tissue engineering. Expression of the antimicrobial peptides such as defensins, elafin and SLPI which are essential elements of the innate immune system results in antibacterial properties of the AM. Preservation is necessary to reach a ready-to-use source of the AM. However, these methods might change the properties of the AM. The aim of this study was to evaluate antibacterial properties of the AM after preservation. Antibacterial property of the fresh AM was compared with cryopreserved and freeze-dried AM by modified disk diffusion method. Staphylococcus aureus ATCC 25923, Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 25922 and two clinical isolated strains of E. coli were cultured in Mueller Hinton agar and a piece of the AM was placed on agar surface. After 24 h incubation, the inhibition zone was measured. In addition, one of the most important antibacterial peptides, elafin, was measured by ELISA assay before and after preservations procedures. Antibacterial properties of the AM were maintained after cryopreservation and freeze-drying. However, the inhibition zone was depending on the bacterial strains. The cryopreservation and freeze-drying procedures significantly decreased elafin which shows that antibacterial property is not limited to the effects of amniotic cells and the other components such as extracellular matrix may contribute in antibacterial effects. The promising results of this study show that the preserved AM is a proper substitute of the fresh AM to be employed in clinical situations.

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Introduction

Amniotic membrane (AM) is the innermost layer of fetal membranes that composes of three layers including epithelial cells, basement membrane and avascular stroma [32]. The AM has been used in transplantation as a biomaterial since 100 years ago [4]. The AM is applied in skin transplantation [7], wound dressing for burned skin [1], healing of chronic leg ulcers [27] and in treatment of acute Stevens-Johnsons syndrome [14]. Moreover, it is widely used in surgical reconstruction of ocular surface [26], bladder, vagina, occlusion of pericardium, prevention of surgical adhesions [34] and vascular tissue engineering [33]. Dressing of burns with the AM can decrease the pain, electrolyte abnormalities and bacterial infection and increase the rate of re-epithelialization in patients [1]. The presence of hyaluronic acid [16] and collagen types I, III, IV, V and VI, fibronectin, elastin, laminin, nidogen and proteoglycans makes the AM as a scaffold for proliferation and differentiation [32,34] and delivery of stem cells [41]. Amniotic cells also express both of the mesenchymal and epithelial stem cell markers [25]. In addition, the AM has anti-inflammatory, low immunogenicity [17] and angiogenic modulatory properties [8,15,31] as well as antibacterial activity [40].

Antibacterial property of the AM prevents the fetal ascending infections [2]. Maternal bacterial infections cause the preterm delivery [12] or fetal and neonatal disease [9,18,19]. The natural antimicrobial molecules in the AM are the components of innate immune system that protect against both Gram-negative and Gram-positive bacteria, fungal and viral infections [10,22]. Whey acidic peptide (WAP) is a group of antimicrobial peptides in the AM which includes elafin (skin derived antileukoproteinase) and secretory leukocyte protease inhibitor (SLPI) [36]. Moreover, the AM contains defensins which are second group of innate immunity peptides divided into two main groups: α-defensin and β-defensin [11]. The α-defensins are found in neutrophils (Human neutrophil peptide; HNP1-4) and the Paneth cells of the small intestine and other epithelial sites (Human defensin; HP5-6) [38,21]. The human β-defensins (HBD1-4) are the main group of antimicrobial peptides...
in mucosal epithelial cells [22]. During the pregnancy, HBD1-3 are up-regulated in placenta and fetal membrane [23]. Human β-defensin3 is frequently expressed in amniotic epithelial cells by exposure to microbial constituents [6]. In addition, the AM contains some natural elements like lactoferrin and interleukin-1 receptor antagonist which reduce the inflammation and contribute to decrease bacterial infection [20].

Since the fresh AM is not available regularly, the preservation is essential to reach a ready-to-use source of the AM for clinical applications. There are some procedures to preserve the AM for long time. Freeze-drying (lyophilization) is one of the preservation processes in which water is removed from a tissue by sublimation [34]. The freeze-dried AM can be stored at room temperature for long periods and its transportation is easy. Another common preservation method of the AM is cryopreservation. In cryopreservation, a cryo-protectant like glycerol or DMSO is used to keep the cell structure in deep freezing (−80 °C or lower). However, preservation methods affect cellular structure and extracellular matrix and might lead to changes in the antibacterial properties of the AM [30]. The aim of this study was to evaluate antibacterial properties of the AM after cryopreservation and freeze-drying.

Materials and methods

Preparation of fetal membrane

The Shahid Beheshti University of Medical Science Institution Research Ethic Committee approved all experiments. Fresh placenta was obtained on sterile condition from healthy women at the time of elective Caesarian. Gestational age was 36–38 weeks of pregnancy and informed consents were obtained from all parents. All of these cases were serologically negative for HIV, HBV, HCV and syphilis. The placenta (n = 30) was transferred on the sterile condition with normal saline at 4 °C. Chorio-amniotic membrane was removed from placenta. Amnion was separated from chorion by peeling under laminar flow and rinsed three times with cold phosphate buffered saline (PBS) to clean all blood remnants (Fig. 1).

Microbial assay

Three standard strains, Staphylococcus aureus ATCC 25923, Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 25922 and two clinically isolated strains of E. coli (T3 and T4) were used for susceptibility tests. At first, these bacteria were cultured on the blood agar or EMB agar (both from Merck, Germany) and incubated at 35 ± 2 °C overnight. Some isolated bacterial colonies were harvested from plates and separately suspended in the sterile normal saline. The turbidity was equivalent to a 0.5 McFarland standard. Finally, these bacteria were cultured on the Muller-Hinton agar plates (Merck, Germany).

The fresh AM was washed with PBS and cut into small pieces (1 × 1 cm). Then each piece of the AM was put on the cultured Muller-Hinton agar plate in accordance to modified disk diffusion method [29,40]. Then, these plates were incubated at 35 ± 2 °C overnight and then inhibition zone was measured. Fresh samples were examined within 4 h after isolation of the placenta.

For freeze-drying, the fresh AM were pre-frozen for 30 min and lyophilized in a freeze-dryer (ModulyoD, Thermo Electron Corporation, USA) at −55 °C (cold trap temperature) for 24 h to prepare the freeze-dried AM. The freeze-dried AM can store in room temperature. For rehydration, the freeze-dried AM was put into the PBS for 2 h. Then rehydrated freeze-dried AM was flattened on the seeded Muller-Hinton agar plats similar to procedure performed in the fresh AM. Subsequently, plates were incubated at 35 ± 2 °C overnight and inhibition zone was measured.

For cryopreservation, the AM was placed in sterile PBS containing 10% dimethylesulphoxide (Me2SO) (Merck, Germany), 10% Dubbelco’s modified Eagle medium (DMEM)/F12 (Gibco, USA), 10% FBS (Gibco, USA) with 8 min equilibrium time and stored rapidly at −80 °C for 6 months. Before using, the cryopreserved AM was thawed at room temperature and rinsed three times in PBS to remove remnants of Me2SO. Then the cryopreserved AM was cut into small pieces (1 × 1 cm) and employed like the fresh AM in the cultured plates and incubated overnight. In order to avoid probable contamination within processing and preparation of the AM, a small piece (1 × 1 cm) of fresh, cryopreserved and freeze-dried AM was put on the Muller-Hinton agar plates and microbial growth was controlled 24 h after cultivation of tissues.

In the present study, AMs were cryopreserved and lyophilized from the same placentas and their anti-bacterial properties were compared with those of the same fresh AM.

ELISA assay

Following the preparation of three forms of the AM (fresh, freeze-dried and cryopreserved), a 5 × 5 cm piece of the AM was minced to smaller pieces and added to 10 ml PBS. Extract was obtained from the AM by sonication with 80 W and 0.5 s cycle for 12 min (hielscher, Ultrasound Technology, Germany). Then tissue residues were removed by centrifuging at 1000 rpm for 4 min. After that, supernatant was collected and centrifuged at 8000 rpm for 5 min. Consequently concentration of the elafin was measured in the deposit by elafin ELISA kit (Abcam, USA) according to company instruction.

Statistical analysis

Statistical comparisons were performed as mean ± standard error of the mean (SEM). Statistical significant was determined by means of one-way analysis of variance (ANOVA) followed by Tukey’s post-test. A P-value less than 0.05 was considered statistically significant.

Results

In this study, we examined antimicrobial activity of the AM against five bacterial strains, S. aureus ATCC 25923, P. aeruginosa
ATCC 27853, *E. coli* ATCC 25922 and two clinically isolated strains of *E. coli* (T3 and T4) by modified disk diffusion method.

The inhibitory effect was appeared under and around the edge of the fresh AM in *P. aeruginosa* ATCC 27853, *E. coli* T3 and *E. coli* T4 after 24 h incubation. Fig. 2 show the inhibitory effect of fresh, freeze-dried and cryopreserved AM in *P. aeruginosa* ATCC 27853, *E. coli* T3 and *E. coli* T4.

Possible microbial contamination of samples were controlled using culture of fresh, cryopreserved and freeze-dried AM on Muller-Hinton agar plates. No growth of bacteria was seen in the cultures; therefore, microbiologically proper samples without bacterial contaminations were used for data interpretation (Fig. 3A).

The inhibitory effects of the AM were also evaluated on *S. aureus* ATCC 25923 and *E. coli* ATCC 25922. In contrast with *P. aeruginosa* ATCC 27853, *E. coli* T3 and *E. coli* T4, the inhibitory effects were not seen in *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 (Fig. 3B and C).

Table 1 shows the number of plates with inhibition zone and elimination of bacterial growth under the AM. Our results show that there is no significant difference between the number of plates with inhibition zone incubated with fresh, cryopreserved and freeze-dried AM (Fig. 4).

The inhibition zones in this study indicate that the inhibitory effects were achieved in the edge of fresh and both preserved (cryopreserved and freeze-dried) tissues. The results regarding the mean and maximum size of inhibition zone have been shown in Table 1. To investigate the effects of preservation on antibacterial properties of the AM, the mean of fresh AM inhibition zone was compared with cryopreserved and freeze-dried AM. The fresh amniotic membrane produced the inhibition zone in *P. aeruginosa* ATCC 27853 and two clinical isolated strains of *E. coli* (T3 and T4). The highest level of inhibition zone was obtained in the *P. aeruginosa* ATCC 27853 (5 mm) which was significantly decreased in *E. coli* (T3 and T4) cultures (*P* < 0.001). In comparison with the fresh AM, no decrease of inhibition zone was seen in the cryopreserved AM except for the *P. aeruginosa* ATCC27853 (*P* < 0.01) (Fig. 5). In the freeze-dried AM samples, the inhibition zone was found after 24 h incubation in *P. aeruginosa* ATCC27853 and two clinical

![Fig. 2](image)

*Fig. 2.* A narrow, but distinct zone of inhibition was appeared in cultures. The arrows show inhibition zones.

![Fig. 3](image)

*Fig. 3.* (A) AM control culture (no colony was found in this culture); (B) *S. aureus* ATCC 25923 cultured with fresh AM; (C): *E. coli* ATCC 25922 cultured with fresh AM (no inhibition zone was found in *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 culture).
The effects of preservation methods on the number of examined plates cultured with three bacterial strains and the mean and maximum size of inhibition zone.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Preservation method</th>
<th>Mean inhibition zone (n=16)</th>
<th>Inhibition zone (n=14)</th>
<th>Max. inhibition zone (n=12)</th>
<th>Mean inhibition zone (n=15)</th>
<th>Inhibition zone (n=15)</th>
<th>Max. inhibition zone (n=12)</th>
<th>Mean inhibition zone (n=11)</th>
<th>Inhibition zone (n=11)</th>
<th>Max. inhibition zone (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa ATCC 27853</td>
<td>Fresh</td>
<td>2.8 mm</td>
<td>1.5 mm</td>
<td>5 mm</td>
<td>7.5 mm</td>
<td>8.4 mm</td>
<td>11 mm</td>
<td>6.5 mm</td>
<td>5.5 mm</td>
<td></td>
</tr>
<tr>
<td>E. coli ATCC 25923</td>
<td>Cryopreservation</td>
<td>0.8 mm</td>
<td>1.0 mm</td>
<td>1.5 mm</td>
<td>0.8 mm</td>
<td>0.5 mm</td>
<td>0.7 mm</td>
<td>1.0 mm</td>
<td>1.0 mm</td>
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<tr>
<td>E. coli strains T3 and T4</td>
<td>Freeze-drying</td>
<td>0.7 mm</td>
<td>0.9 mm</td>
<td>1.3 mm</td>
<td>1.2 mm</td>
<td>0.8 mm</td>
<td>1.0 mm</td>
<td>0.7 mm</td>
<td>1.0 mm</td>
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</table>

Discussion

The antibacterial property is one of the advantages of the AM which makes it as an appropriate matrix for clinical applications [24,39,40]. However, owing to the limitations of the fresh biomaterials and concerns regarding window period infections, it is essential to preserve the AM. On the other hand, preservation procedures could change some characteristics of the AM [30]. To the best of our knowledge, the effects of preservation methods on antibacterial activity of the AM have not been reported previously. In this study, we evaluated antibacterial properties of the AM after cryopreservation and freeze-drying and compared with those of the fresh AM.

Based on the results of this study, antibacterial effects of the AM were seen on P. aeruginosa ATCC 27853 and two clinically isolated strains of E. coli (T3 and T4). In contrast, inhibitory effects of AM were not observed on S. aureus ATCC 25923 and E. coli ATCC 25922. These results show that antibacterial effect of the AM is depending on bacterial genus and strain, which are in consistent with the reports of Kjaergaard et al. They demonstrated that the narrow inhibition zone (about 1 mm) was appeared in Group A streptococcus and Staphylococcus saprophyticus [24].

The maximum inhibition zone in this study was belonging to P. aeruginosa ATCC 27853 (about 5 mm). The different inhibition zones and strain dependent effects of the AM can be attributed to the bacterial ingredients which can be inhibited by the AM [37]. For instance, elafin as an antibacterial peptide in the AM inhibits serine peptidase, a virulence factor in P. aeruginosa [5]. Hence, more inhibition zone in P. aeruginosa may be due to inhibition of bacterial serine peptidase as a specific mechanism along with the other known antibacterial mechanisms of the AM.

The main aim of this study was to compare antibacterial effects of the AM after cryopreservation and freeze-drying. The cryopreservation and freeze-drying are two common methods for preservation of the AM. Our results show that the cryopreserved and freeze-dried AM have the same inhibitory effects on bacterial growth. The antibacterial property of the AM has a critical role in application of the AM in burn treatment [28] and ophthalmological interventions [13]. Therefore, antibacterial property is necessary to be maintained in the AM after cryopreservation and freeze-drying. However, further studies will be required to evaluate in vivo antibacterial property of the preserved AM.

Although less than 50% of the amniotic epithelial cells were alive after cryopreservation [30], the same results were achieved in cryopreserved AM. In the same manner, there was no viable cell after freeze-drying of the AM and the cells were removed in some areas of the amniotic epithelium [35], while antibacterial effects were maintained in freeze-dried AM. It seems ingredients involved in antibacterial effect of the AM are not limited to the amniotic cell contents and the other components may contribute in antibacterial effects of the AM. Antibacterial peptides residues in the basement membrane such as lactoferrin [20] and extracellular matrix
Components can be responsible for antibacterial effects of the cryopreserved and freeze-dried AM. For example, it has been shown that hyaluronic acid (in extracellular matrix of the AM) inhibits dose-dependently growth in Enterococci, Streptococcus mutans, E. coli and P. aeruginosa [3].

As mentioned in the results, the cryopreserved AM was rinsed three times in PBS to remove remnants of DMSO. Furthermore, the freeze-dried AM was put into the PBS for 2 h for rehydration and absorbed water more than 10 times its weight [30]. Therefore, the decrease in antimicrobial properties of cryopreserved and freeze-dried AM could be likely because of rinsing and rehydration process.

Since elafin is one of the main antibacterial peptides in the AM [23], the concentration of it was examined before and after preservation. The results showed that cryopreservation and freeze-drying procedures significantly decreased elafin in comparison with fresh AM. Decrease of inhibition zone in P. aeruginosa ATCC27853 in comparison with the fresh AM can be ascribed to decrease of elafin during preservation process. As described, growth of P. aeruginosa is depending on serine peptidase which is inhibited by elafin [5].

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**Fig. 4.** The percent of plates with inhibition zone in which the effects of fresh, cryopreserved and freeze-dried amniotic membrane were evaluated on three bacterial strains.

**Fig. 5.** The mean of inhibition zone (mm) was appeared in P. aeruginosa, E. coli T3 and T4 after 24 h incubation with fresh, cryopreserved and freeze-dried AM.

**Fig. 6.** Comparison of elafin concentrations in fresh, cryopreserved and freeze-dried amniotic membrane by ELISA kit.
Conclusion
The results of this study show that antibacterial property of AM was maintained after cryopreservation and freeze-drying. However, antibacterial effect of preserved AM is depending on bacterial genus and strain, which is possibly because of different amount of antibacterial peptides in amniotic cells or the other parts of the AM e.g. extracellular matrix ingredients. Further studies are necessary to investigate the other antimicrobial elements in the AM and antibacterial effects of the AM on the other bacterial strains which are more prevalent in clinical situations.

Conflict of interest
The authors declare no conflict of interest.

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References