Original Article

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The Antibacterial Effect of Low Temperature Stored Amnion on Growth of Escherichia Coli, Staphylococcus Aureus and Pseudomonas Aeruginosa

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ABSTRACT

BACKGROUND AND OBJECTIVE: Amniotic membrane (AM) has a lot of applied properties like anti-bacterial characteristic mediated by peptides such as elafin. Because of limitations in use of freshly prepared tissue, there are various methods for long-term preservation of amniotic membrane. This study was conducted to determine the effect of cryopreservation, as one of the common methods of preservation of amniotic membrane, on its antibacterial property against the growth of commonly occurring bacteria in the clinic.

METHODS: In this experimental study, the effect of fresh AM (from elective Cesarean) and cryopreserved (by 10% DMSO) AM on the growth of three standard bacterial strains including Escherichia coli ATCC 25922, Staphylococcus aureus, Pseudomonas aeruginosa and two clinical isolated strains of E.coli were evaluated using disk diffusion test. In this method, pieces of fresh or cryopreserved AM was placed in the culture plate after bacterial culturing. After incubation, the number of plates with inhibition zone and amount of inhibition zone were measured. The amount of elafin was measured in AM samples using ELISA.

RESULTS: Fresh AM inhibit the growth of Pseudomonas aeruginosa and two clinical isolated strains of E.coli. However, it has no effect on the growth of standard strain of Escherichia coli and Staphylococcus aureus strain. There is no difference in the number of plates including inhibition zone between fresh and cryopreserved AM. The amount of elafin decreased significantly in cryopreserved AM ($p<0.01$).

CONCLUSION: The results of this study showed that the anti-bacterial property of the AM depends on bacterial species. In addition, the cryopreservation process maintains anti-bacterial properties of amniotic stem cells.

KEY WORDS: *Amniotic membrane, Anti-bacterial, Cryopreservation, Bacterial strain.*

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Introduction

Over the years, embryonic membranes have been one of the substitute tissues used in surgery (1-3). The amniotic membrane is the innermost layer of the embryonic membrane consisting of five layers of epithelial layer, basal membrane, inner compact layer, fibroblastic layer and outermost spongy layer, and has unique properties (4). This tissue can affect angiogenesis (5), induces apoptosis (6) and reduces inflammatory and immune responses (7, 8). Also, having antimicrobial properties can inhibit the growth of bacteria, viruses and fungi (9, 10).

Cellular matrix compounds in the basal membrane of the amniotic membrane are a natural scaffold for cell culture and use in tissue engineering (11, 12). Amniotic membranes contain a large number of human stem cells that can be used widely. Access to this tissue is easy and there are no moral issues for use (13, 14). One of the important properties of amniotic membrane is its antimicrobial property, which has many clinical applications (15).

Antimicrobial properties of amniotic membrane are due to antimicrobial peptides, including Defensins, SLPI , and Elafin. These molecules are produced and secreted at mucosal surfaces and often by epithelial cells. "Whey acidic peptide (WAP)" is a group of antimicrobial peptides in amniotic membranes that include Elafin and SLPIs (16). These molecules are low-weight and have anti-protease activity (16, 17) and elastase inhibition (18-20), and as components of the innate immune system, controlling the inflammatory response at mucosal surfaces, and protect the surface associated with infection. (20). Human Beta-Defensins are another group of peptides that contribute to the development of immune responses to amniotic membrane (17). One of the common methods of using amniotic membrane in the clinic is the use of fresh tissue that is immediately isolated after delivery. One of the limitations of using the fresh amniotic membrane is its rapid destruction. The cells in this tissue survive shortly after delivery and, in addition, due to the presence of latency period, it is possible to transmit some diseases when using fresh tissue. Therefore, methods have been proposed to preserve the amniotic membrane. Cryopreservation is one of the common methods for long-term amniotic membrane maintenance. In this method, using different concentrations of preservatives such as glycerol and Dimethyl sulfoxide, tissue can be stored for months at temperatures of -80°C or -196°C.

Although cryopreservation can affect its properties by affecting the cells and tissue structure of the amniotic membrane (21, 22) due to the limited access to fresh tissue it is necessary to investigate the effect of maintenance methods on the properties of amniotic membrane. In this study, the effect of Cryopreservation on antibacterial properties of amniotic membrane has been investigated.

Methods

This experimental study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences by code of: IR.SBMU. MSP.REC.1394.139 In order to isolate and prepare the amniotic membrane, the placenta tissue obtained from elective cesarean section of healthy women without antibiotic use during pregnancy during 38-40 weeks of pregnancy was prepared from the Erfan Hospital in Tehran under sterile conditions. The placenta samples were transferred to the lab in a container containing sterile saline phosphate buffer at temperature of 4°C. All stages of isolation and preparation of amniotic membrane samples were performed in sterile conditions. The amniotic membrane was separated mechanically from the chorion and washed several times with cold saline phosphate buffer to remove the remaining blood, so that there was no trace of blood stains on it. In order to cryopreservation of amniotic membrane, samples of this tissue after isolation and washing, were stored at -80 °C for 6 months in sterile saline phosphate buffer containing 10% dimethyl sulfoxide (Merck, Germany) and 10% Bovine serum (Gibco, USA) and 10% Dubbelco's modified Eagle medium (DMEM)/F12 (Gibco, USA).

After this period, the samples were gotten out from frozen at room temperature and were washed three times with sterile saline phosphate buffer and then cut to pieces of about 1 cm2. In order to study the antbacterial effects of amniotic membrane, direct disk diffusion method was used on 3 standard strains including Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 25923, Pseudomonas aeruginosa ATCC 27853 and two clinical strains of E. coli T3 and E. coli T4). Clinical strains were prepared from patients of Ayatollah Taleghani Hospital in Tehran. Standard strains were selected according to the guidelines of the Standard Institute of Laboratory and Clinical; meanwhile, all stages of the work were carried out in accordance with the terms of this protocol (23). The

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bacteria were first cultured on blood agar (Merck) as a common culture medium and incubated for 24 hours at 37°C. Isolated colonies were prepared with 0.5 McFarland concentration (1.5×108×CFU) in normal saline and were cultured on the muller Hinton agar (Merck), as a selectable medium. Then, the pieces made from fresh amniotic membrane and cryopreserved AM were placed in bacterial culture plates and the plates were incubated for 24 hours at 37 ° C. After this time, the size of the inhibition zone was measured in all the plates (in millimeters) and the amount of inhibition zone around the amniotic membrane was compared. One sample of each tissue was placed on a plate containing Muller Hinton Agar as a control group to determine the probability of contamination of the amniotic membrane after maintenance. After preparing the amniotic membrane, a 5×5 cm piece of fresh tissue and cryopreserved AM was isolated and, after crushing, 10 ml of normal saline buffer was added. Tissue extracts were obtained using a 80-watt sonication with 0.5 second intervals of 12 minutes. The samples were then centrifuged at 1000 rpm for 5 minutes. The collected supernatant was then centrifuged for 2 minutes at 8000 rpm.

Finally, the amount of Elafin (in pg/ml) in the supernatant was measured using an ELISA kit (Abcam, USA) based on the manufacturer's instructions to compare the amount of Elafin present in the fresh tissue and cryopreserved AM supernatant. The results are reported as Mean±SEM. For statistical analysis, GraphPad Prism software version 5.04 was used and one-way ANOVA and Tukey test were used and $p<0.05$ was considered significant.

Results

In this study, antibacterial activity of fresh amniotic membrane and cryopreserved AM on 5 strains of bacteria including Staphylococcus aureus ATCC 25923, Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 25922 and two clinical strains of Escherichia coli (T3, T4) were investigated by disc diffusion method. An inhibitory effect was observed below and around the edges of the fresh amniotic membrane in Pseudomonas aeruginosa ATCC 27853 and two clinical strains of E. coli (T3, T4) after 24 hours incubation (Fig 1a,b,c). This inhibitory effect was also seen in cryopreserved AM. Inhibitory effect of amniotic membrane stored at -80 °C in Pseudomonas aeruginosa ATCC 27853 and two clinical strains of E. coli (T3, T4) are shown in Figs. 1D, 1E and 1F. No growth was seen in the control group (Fig 1G). Unlike other tested strains, there was no inhibitory effect on Staphylococcus aureus ATCC 25923 and Escherichia coli ATCC 25922 strains (Fig H, I).

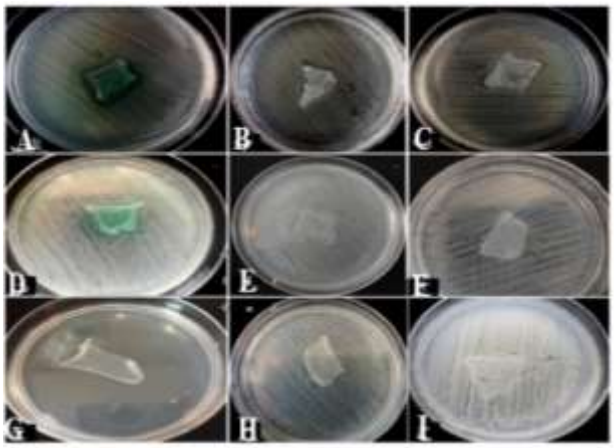

Figure 1. (A, B and C) The formation no growth inhibition zone in the vicinity of the fresh AM: a) Pseudomonas aeruginosa ATCC 27853; b) Escherichia coli T3; c) Escherichia coli T4. (T, E and C) The formation inhibition zone in the vicinity of the Cryopreserved AM: d) Pseudomonas aeruginosa ATCC 27853; E) E3; E3) Escherichia coli T4. (C) Cultivation of AM control. (No colony was found.). G) Escherichia coli ATCC25922 cultivated with fresh AM; (h) Staphylococcus aureus ATCC25923 cultivated with fresh AM. (No growth inhibition zone was observed in staphylococcus aureus ATCC25923 and E. coli ATCC25922)

The number of plates with no growth inhibition zone under the amniotic membrane showed no significant difference between fresh tissues and cryopreserved tissue (Table 1) (Fig 2a). The growth inhibition zone around the edges of cryopreserved AM was similar to the fresh tissue in this study. The fresh amniotic membrane that cultured in the plate with Pseudomonas aeruginosa and the two clinical strains of E. coli (T3, T4) formed the inhibition zone. The highest inhibition growth zones created by fresh tissue in a Pseudomonas aeruginosa cultured plate was 5±1.1 mm, which significantly decreased in E. coli (T3, T4) cultures ($p<0.001$). In addition, the size of the inhibition zone in cryopreserved AM was significantly reduced in comparison with fresh samples in pseudomonas aeruginosa cultures ($p<0.01$), which, was not observed in Escherichia coli (T3, T4) (Fig. 2b). The results showed that the amount of Elafin in fresh tissue was significantly higher than cryopreserved tissue (Table 2) ($p<0.01$) (Fig. 2c).

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Table 1. Comparison of antibacterial effects in cryopreserved tissue with fresh one

Bacterial strain	Cryopreserved tissue		Fresh tissue	
Pseudomonas aeruginosa ATCC 27853	growth retardation under the tissue ✦ (n=11) ⋆7 ،⋆4	**no growth inhibition zone** ⮝ (n=11) 11	growth retardation under the tissue ✦ (n=14) ⋆2 ،⋆12	no growth inhibition zone ⮝ (n=14) 14
Escherichia coli T3	growth retardation under the tissue ✦ (n=11) ⋆7 ،⋆4	no growth inhibition zone ⮝ (n=12) ⋆7 ،⋆5	growth retardation under the tissue ✦ (n=19) ⋆14 ،⋆5	no growth inhibition zone ⮝ (n=21) ⋆19 ،⋆2
Escherichia coli T4	growth retardation under the tissue ✦(n=11) ⋆7 ،⋆4	no growth inhibition zone ⮝ (n=11) ⋆6 ،⋆5	growth retardation under the tissue ✦ (n=15) ⋆12 ،⋆3	no growth inhibition (n=14) zone ⮝ ⋆12 ،⋆2

⮝The number of studied plates in terms of a growth retardation under the tissue, ✦ the number of studied plates in terms of no growth inhibition zone

★The number of plates with no growth inhibition zone, ✶ The number of plates with growth inhibition zone

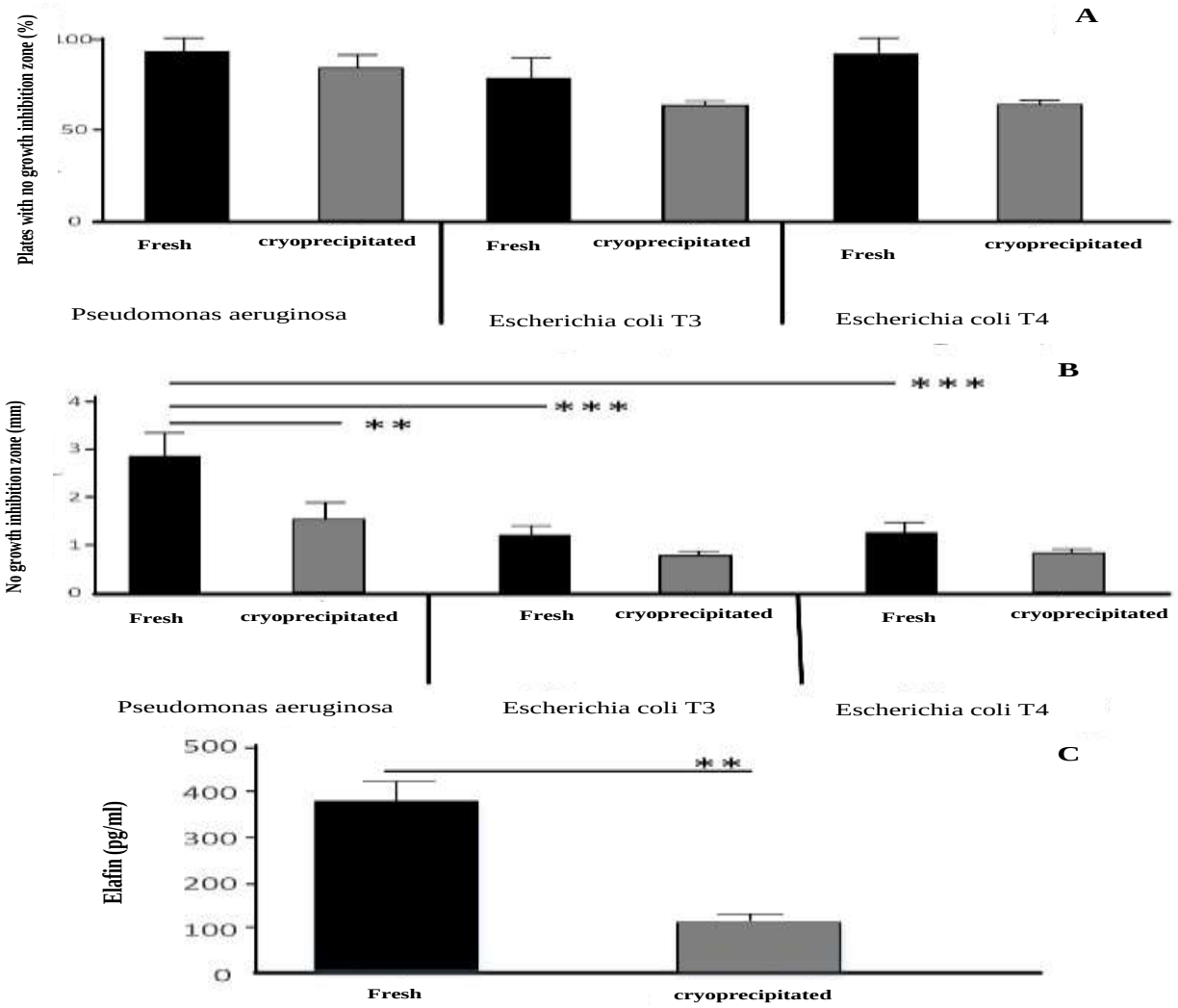

Figure 2. (a) Comparison of the percentage of plates with no growth inhibition zone to the total number of plates around the fresh and cryoprecipitated AM based on the cultivated bacterial species. (B) The mean size of no growth inhibition zone in millimeters (mm) in Pseudomonas aeruginosa and Escherichia coli T3 and T4 appeared 24 hours after incubation in vicinity to the fresh and cryoprecipitated AM (p<0.01 and ***p<0.001). (C) The amount of oligin present in the fresh and cryoprecipitated AM (**p<0.01)**

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Table 2. Effect of processing methods on mean and maximum of no growth inhibition zone in three strains of bacteria

Group	Cryopreserved tissue		fresh tissue	
	Max of inhibition zone (mm)	Mean of inhibition zone (mm)	Max of inhibition zone (mm)	Mean of inhibition zone (mm)
Pseudomonas aeruginosa ATCC 27853	3	1.5(n=11)	5	2.8(n=14)
Escherichia coli T3	1	0.8(n=12)	2	1.2(n=21)
Escherichia coli T4	1	0.9(n=11)	2	1.3(n=14)

Discussion

Based on the results of this study, the fresh amniotic membrane in plates with Pseudomonas aeruginosa ATCC 27853 and the two clinical strains of E. coli T3 and T4 caused a inhibition growth zone. However, there was no inhibitory effect on the growth of Staphylococcus aureus ATCC25923 and E. coli ATCC25922 below and around the amniotic membrane. These results indicate that the antibacterial effect of amniotic membrane is dependent on the genus and strain of bacteria that confirmed by Kjaegaarda et al. Kjaegarda et al. have reported the existence of a narrow inhibition zone (about 1 mm) around the amniotic membrane in the vicinity of group A Streptococcus and Streptococcus saprophyticus (9). The largest extent of the inhibition growth zone in this study was related to Pseudomonas aeruginosa (about 5 mm). Differences in the amount of inhibition growth zone and the type of bacteria can be attributed to the different structure of the bacteria (24). For example, Elafin, which is an amniotic antimicrobial peptide, inhibits one of the virulence factors of Pseudomonas aeruginosa called serine peptidase (25). Therefore, it seems that the effect of this tissue on pseudomonas aeruginosa was more pronounced.

Although antibacterial effects of tissues such as amniotic and chorion (26), which are of great importance in the treatment of burns (27) and ocular diseases (28), have been shown in previous studies, so far little studies have been done on the effect of the preserving method on the properties of these tissues. The results showed that the cryopreservation of amniotic membrane significantly reduced the amount of Elafin compared to fresh tissue. In previous studies, it has been shown that the viability of amniotic membrane epithelial cells is reduced by about 50% after freezing (21, 29), despite decreasing the cellular viability and reducing the amount of Elafin, is still retained by the amniotic membrane antibacterial properties. Therefore, in addition to secretion peptides from amniotic membrane cells, other factors can also contribute to its antibacterial properties. Antimicrobial peptides in the amniotic membrane, such as lactoferrin (30) and extracellular matrix components, can contribute to the anti-bacterial property of cryopreserved tissue. For example, hyaluronic acid in the extracellular matrix of amnion can inhibit Enterococci, Streptococcus mutans, two strains of Escherichia coli and Pseudomonas aeruginosa depending on the growth concentration (30). The results of this study showed that cryopreservation maintenance method was able to maintain the antibacterial properties of amniotic membrane and antibacterial properties of amniotic membrane was dependent on bacterial strain that could allow the use of cryopreserved AM in the clinic. Further studies are needed to investigate the antimicrobial properties of amniotic membrane and its clinical applications.

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