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Antimicrobial effects of amniotic membrane on some bacterial strains

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Abstract

Introduction: Antibiotic resistance in clinical aspect is a concern. The human amniotic membrane (AM) has antimicrobial effects mostly because of peptides such as alafin and HBDs.
Objectives: The aim of this study was to investigate antimicrobial effects of AM on some standard strains of *Pseudomonas aeruginosa* ATCC27853, *Salmonella enterica* BAA-708, *Escherichia coli* ATCC25922, *Klebsiella pneumonia* ATCC7881, *Enterococcus faecalis* ATCC29212 and *in vivo* study on *Pseudomonas* keratitis.
Materials and Methods: AM with and without antibiotic cocktail were prepared from Imam Khomeini hospital of Tehran and cut into 1.5×1.5 cm. The 0.5 McFarland suspension of strains was prepared and cultured on plate media and then both types of membranes were put on cultures. The effect of environmental factors (temperature, time and pH) was considered. In laboratory animal model, keratitis was made in 14 rabbits and they were divided into two control and AM groups. The membrane was bound to the control group. The infiltration wound diameter was measured.
Results: The growth inhibition by cocktail membrane occurred in 5 strains, but membrane without cocktail could not inhibit the growth of *K. pneumonia* and *E. faecalis in vitro*. Time and pH conferred no antimicrobial effects, while temperature showed an effect on *P. aeruginosa*. The decrease in the infiltration in the membrane group was significant compared to the control group $P<0.05$).
Conclusion: The AM has antimicrobial properties and can be used alongside antibiotics for the treatment purposes.

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Introduction

Bacterial keratitis is a menace and concern in the optical aspects and considering antibiotic resistance development in bacterial agents, substitution or combination therapy may be essential (1). If untreated, cornea infection may develop towards perforation and consequently glaucoma, cataract and endophthalmitis may occur as the outcomes (2,3). The most common agents are *Pseudomonas aeruginosa*, *Escherichia coli*, Group A streptococci and *Staphylococcus aureus*. *P. aeruginosa* is a very common (up to 70%) agent in the cornea infection (1,4,5). Infection and production of degrading enzymes by these species play a critical role in the invasion and destroying cornea cells (6). On the other hand, bacterial cell wall compounds interact with Toll-like receptors and initiate an inflammatory response via production of cytokines and chemokine molecules. The subsequent inflammatory response induces the cornea tissue damage. Furthermore, polymorphonuclear leukocytes cells (PMNs) migration and release of lysosome and oxidative compounds play an important role in the bacterial eradication. These responses may cause cornea tissue damage as well (7). Severe bacterial keratitis needs rapid and extended use of local antibiotic therapy.

Key point

The amniotic membrane has antimicrobial properties and can be used alongside antibiotics for the treatment purposes.

However, these compounds are toxic for the epithelial cells and have potential of harm to these cells after a period of consumption. The antibiotic resistance has made many difficulties for treatment of these infections. The human amniotic membrane (AM) is the inner layer of placenta and is composed of three layers including cuboidal epithelium, thin stem membrane and stromal matrix (8). Several studies have shown that both AM and liquid exhibit the antimicrobial and wound healing properties. Surveys have revealed the presence of natural antimicrobial peptides including alafin, SLPI and 1-3HBD in the epithelium of the AM (9-12). The antimicrobial effects of AM have been demonstrated against several species such as *E. coli,* Group A streptococci, *P. aeruginosa* and *S. aureus* (13-16). AM graft for epithelial reformation has been employed in order to eradicate the *P. aeruginosa* infection of keratitis and have exhibited desirable outcomes (17).

Objectives

The aim of the current study was to assess antimicrobial properties of AM against *P. aeruginosa* keratitis in vivo and over some other standard strains in vitro.

Materials and Methods

Bacterial strains

Five strains including *P. aeruginosa* ATCC27853 (from Shiraz University of Medical Sciences, causing keratitis), *S. enterica* BAA-708, *E. coli* ATCC25922, *K. pneumonia* ATCC7881, *E. faecalis* ATCC29212 (from Tehran University of Medical Sciences) were prepared for antimicrobial testing. The half McFarland of these strains was prepared.

Human amniotic membrane cutting and susceptibility testing

The human AM cuts were prepared in fresh from (with and without cocktails) the organ transplant, Imam Khomeini hospital bank in Tehran. The specimens were from placenta of women experiencing cesarean and negative for HIV, HBV, HCV and syphilis agents. Under sterile conditions, washing was done with normal saline and in next step with cocktails of cloxacillin 50 ug/mL, streptomycin 50 ug/mL and amphotericin B 2/5 ug/mL. The specimens with and without antibiotics containing an equal volume of ice were transmitted to the health department of Medical Microbiology of Tehran University of Medical Sciences. For the antimicrobial susceptibility testing, the cut sizes were 1.5×1.5 and were placed on center of Muller-Hinton agar after bacterial culture similar to susceptibility testing protocol. A Para film was used as control negative and for ensuring the sterility, a medium without bacterial culture was used. After 24-hour incubation, the diameter of four directions was measured and the mean was obtained.

Environmental factors effects

The effects of temperature, pH and time on the antimicrobial effect of AM were assessed. In case of time effects, the diameters of growth inhibition were measured after 24, 48 and 72 hours of incubation. For pH effect, the susceptibility was evaluated in media with three different pH including 6.5, 7 and 7.5. Furthermore, for temperature effect, the plates were incubated in 25°C, 33°C and 37°C.

Animal model study of amniotic membrane effect

Eighteen polish rabbits with 1.5-2 kg were prepared from Razi Institute of Karaj, Iran. After one-week isolation, the rabbits were anesthetized and 0.5 cc of half McFarland of *P. aeruginosa* was injected into the stromal cornea using a 30G syringe attached to a micro syringe and by utilizing a biomicroscope to make keratitis after 20 hours. For membrane graft, after keratitis was observed, the rabbits were divided in two amniotic membrane transplantation (AMT) and control groups. The AMT group was anesthetized again and the AM was bound to the keratitis eyes using nylon sutures in direction of epithelium side following the sterile conditions by an optometrist (18,19).

Microscopic image capture and observation of results

Clinical observations of *Pseudomonas* keratitis and also corneal turbidity, epithelial impairs, descemetocele (protrusion of Descemet's membrane through the cornea), infiltration in stromal area and image capture before and after the membrane graft were measured in first, third and seventh days. The ulcer diameter of infiltration (due to the epithelial cells secretions) in stromal cornea which is the diagnostic criteria of effects of AM in this study was measured using ImageJ (version 1/44). The mean measurements of the numbers were obtained in each day of measuring. Moreover, the infiltration was compared between the AMT and the control groups.

Ethical issues

The research followed the tenets of the Declaration of Helsinki. Consent for operation and study had been taken. The ethical committee of Tehran University of Medical Sciences approved the research. All patients' information remained confidential. This study has the Iranian registry of clinical trials of IRCT 2015248N9.

Data analysis

The data were analyzed using SPSS version 16. Repeated measure analysis variance test was applied for comparison of means across one or more variables. *P* values less than 0.05 were considered as a significant result.

Results

Amniotic membrane antimicrobial effects

The inhibition zone was measured for *P. aeruginosa*, *S. enterica* and *E. coli* strains, but not observed in case of *E. faecalis* and *K. pneumonia* and even the colonies were observed under the membrane (Figures 1 and 2; Table 1).

Effects of environmental factors

The results of temperature, bacterial concentration, time

Table 1. The growth inhibitory zones against bacterial strains

Bacterial strains	*E. coli*	*S. enterica*	*P. aeruginosa*	*E. faecalis*	*K. pneumonia*	*P* value
Growth inhibition zone (mm)	50	63	2	0	0	0.001

The inhibition zone was observed among all 5 strains by using membrane with antibiotic cocktail.

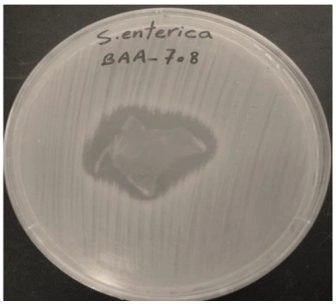

Figure 1. The inhibitory zone of amniotic membrane against *S. enterica* BAA-708

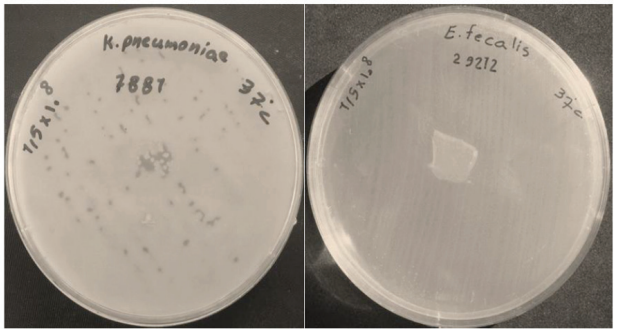

Figure 2. The resistance of *K. pneumonia* and *E. faecalis* to the amniotic membrane inhibitory compounds.

and pH variations effects on the inhibitory effect of AM showed no significant difference for all 5 strains when alterations fulfilled.

Animal model keratitis inhibitory effects

The corneal keratitis results were observed in both groups before membrane graft and 20 hours after injection of *P. aeruginosa*, including tear secretions, photophobia and conjunctiva hyperemia without microscope employment. The biologic microscopic observations included cornea inflammation, stromal ring infiltration and corneal turbidity (Table 2 and Figur 3).

Discussion

In the present study, the antimicrobial effect of AM was shown against three strains including *P. aeruginosa* ATCC27853, *S. enterica* BAA-708, *E. coli* ATCC25922, but *K. pneumonia* ATCC7881, *E. faecalis* ATCC29212 showed resistance to it. In the study by Kjaergaard et al, similarly the amniotic and carrion membranes exhibited inhibitory effect against group A and B streptococci, *S. aureus, S. saprophyticus* and *E. faecalis* in vitro, but in contrast their study demonstrated a narrow effect against *E. coli* and *P. aeruginosa* (13,14). Similar to the present study, in another study by Chakraborty and Bhattacharyya, the human amniotic extract exhibited a great inhibitory effect on *E. coli* DH5α (15). In the current study, similar to other previous data, the cesarean AM was used because of lower load of contamination. It has been noted that AM contains other components such as lysozyme, transferrin, immunoglubolin7S, β1 and β1a globulins and several others that can inhibit bacterial growth, and thus it should be carefully washed with sterile normal saline (20). It has been proposed that AM is a reservoir of antimicrobials transferring them to the cornea in a period of time without harm to the epithelium.

In this study, the increase in inhibition zone in three susceptible strains using antibiotic cocktail is capable of diffusing the antibiotic from the surface with antibiotic to the surface without it (21,22).

Almajano et al showed that the environmental factors and conditions interfered with the antimicrobial effects of AM. The pH effect on polyphenol antimicrobial properties led to the control of food contamination (23). However, in this study unlikely the pH showed no increasing effect on AM effects against susceptible strains. The alterations of minimum inhibitory and minimum bactericidal

Table 2. Three groups tested in this study

Groups/Days		1	3	7	*P* value
Control		Slight infiltration and corneal turbidity, photophobia and tear secretions	Increased infiltration and corneal turbidity, epithelial impairs, without descemetocele and perforation	Increased infiltration and corneal turbidity, epithelial impairs, descemetocele and perforation in 4 of 7 rabbits	0.021
AMT		Infiltration and corneal turbidity and epithelium impairs	Increased infiltration and corneal turbidity	Expanded infiltration and corneal turbidity without descemetocele and perforation	0.011
Ulcer diameter (mm)	Control	2.25	7.01	23.75	0.001
	AMT	2.62	6.25	8.02	0.26

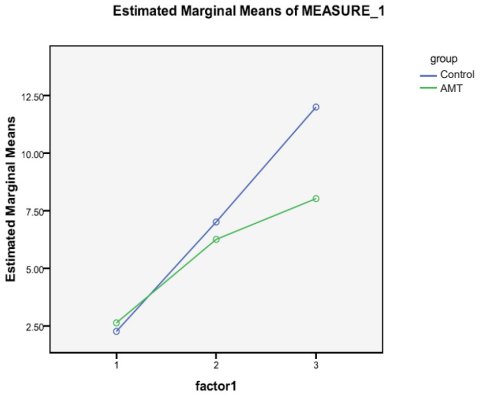

Figure 3. Comparison of AMT and control groups with regard to the infiltration lesion diameter (mm) during 3 different days (1st, 3rd and 7th) of study.

concentrations (MIC and MBC, respectively) in different temperatures have been shown in case of methicillin and penicillin- resistant *S. aureus* and *E. faecalis* (24,25). In this study, the incubation of plates in 25°C and 33°C instead of 37°C showed an increased activity against *P. aeruginosa*.

In this study, the time duration of incubation showed no increasing effect on the inhibitory properties of AM. Similarly, Talmi et al showed that AM incubation time in 24°C, 48°C and 72°C exhibited no different inhibitory results. The similar effect of AM in different environmental conditions suggests that related antimicrobial peptides are persistent in these conditions.

In this study two bacterial dilutions including 1.5×10^6 and 1.5×10^7 demonstrated no significant difference in the inhibitory zone in any of strains. Similarly in the study by Talmi, use of 3×10^6 and 3×10^8 dilutions of group A streptococci exhibited no difference in inhibitory zone.

In the animal model study, AM graft could significantly decrease the keratitis signs. It has been shown that AM can remove bacterial effects on burn patients and is ideal burn wound dressing (26). Tehrani et al found that the preserved AM is a suitable substitute for the fresh AM in clinical situations (27). Obviously, Parthasarathy et al highlighted the presence of a potential antimicrobial activity of both amniotic and chorionic membranes to inhibit several bacterial and fungal pathogens in which maximum activity was recorded by AM (28).

Conclusion

The findings of this study showed that AM is an effective factor against bacterial strains in vitro and also demonstrated keratitis healing by decreasing the signs of descemetocele and perforation of corneal tissue.

Authors' contribution

FF and VN conducted the research and contributed to the manuscript equally. All authors read and signed the final paper.

Conflicts of interest

The authors declare that there are no competing interests.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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