Full Length Research Paper

Epithelium of human fresh amniotic membrane has antimicrobial effects in vitro

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This study was carried out to investigate the antimicrobial effects of human fresh amniotic membrane and human amniotic homogenate supernatant (HAHS) in vitro. Fresh human amniotic membranes were harvested and used to make a cellular amniotic membranes (fresh amniotic membranes without the epithelium) and HAHS. Then, the antimicrobial effects of both the fresh human amniotic membrane with the epithelium and fresh amniotic membranes without the epithelium were observed. The antimicrobial effects of fresh HAHS were examined with Kirby-Bauer method. The antimicrobial capacity was compared with ten antibiotics with the Broth Micro-dilution Method, and the impacts of temperatures and pH values and preserved time of HAHS on the antimicrobial capacity were investigated. Finally, transmission electron microscopy (TEM) was used to explore the possible mechanism of the antibiosis of HAHS. Human fresh amniotic membrane with the epithelium had antimicrobial effects, while fresh amniotic membrane without the epithelium did not have any antimicrobial activities. HAHS showed antimicrobial activities for the three standard strains: Staphylococcus aureus ATCC, Escherichia coli, Pseudomonas aeruginosa; four clinically isolated sensitive bacterial strains: Streptococcus pneumonia, Staphylococcus epidermidis, Staphylococcus haemolyticus, and Proteus mirabilis; three clinically isolated fungal strains: Blastomyces albicans, Fusarium solani, Aspergillus fumigatus; and one clinically isolated multi-drug-resistant strain: Enterococcus faecalis. The minimum inhibitory concentration (MIC) of HAHS for Staphylococcus aureus was less than that of Chloramphenicol, equal to that of Clindamycin, greater than that of Penicillin, Rifampicin, and Levofoxacin. Fresh amniotic membrane with epithelium and HAHS had antimicrobial activities, and fresh amniotic membrane without the epithelium did not, indicating that antimicrobial activities are related with the epithelium. HAHS had broad antimicrobial activities and stable characteristics. The mechanism of antimicrobial effect of HAHS was probably interacting with the plasma membrane of the bacteria.

Key words: Human amniotic membrane, antimicrobial effects, amniotic homogenate supernatant, minimum inhibitory concentration, minimal bactericidal concentration.

INTRODUCTION

Amniotic membrane is a biological material and is easy to obtain. Human amniotic membrane is smooth and has no blood vessels, low antigenicity and amniotic membrane transplantation surgery has been a focus in ocular surface reconstruction in recent years. The main effects of amniotic membrane include accelerating epithelization, maintaining normal epithelial phenotype, decreasing inflammatory responses and mitigating vascularization (Seo et al., 2008). The antimicrobial action of amniotic fluid has been proved (Thadepalli et al., 1978), however, the part of the human amniotic membrane having this antimicrobial effects is not clear (Talmi et al., 1991; Kjaergaard et al., 1999, 2001). In the present study, the antimicrobial effect of the epithelium of the human fresh amniotic membrane was investigated.

MATERIALS AND METHODS

Main reagents, drugs and equipment

Mueller-Hinton (MH) broth (Land Bridge Technology Co., Beijing,
China, 7 mm Colombia blood flat (Pang Tong Co., Chongqing, China), Pancreatin powder, EDTA powder (Sigma-Aldrich Co., St. Louis, MO, USA), DME / Ham ’s F12 powder (Thermo Scientific HyClone Co., UT, USA), BCA Protein Assay box (Fierce Biotechnology, Rockford, IL, USA), 10 kinds of antibiotic standard products, including Penicillin, Clindamycin, Chloramphenicol, Rifampicin, Levofloxacin, Gentamycin, Tobramycin, Sulfacetamide Sodium, Cefuroxime Sodium, and Norfloxacin (Drug and Biological Products Testing Institute, Beijing, China), 96 hole plate (CoStar Group Inc., NY, USA), homogenate machine (Zhejiang Machinery Factory, Hangzhou, China), JY92-II ultrasonic cell pulverizer (Xinzhi Biotechnology Research Institute, Shanghai, China), ELISA reader and protein nucleic acid analyzer (Bio-Rad Laboratories, Inc., CA, USA), air shaker (Thermo Forma, OH, USA), 5804R refrigerated centrifuge (Eppendorf, Hamburg, GERMANY).

Microorganisms

In this study, three standard bacterial strains: Staphylococcus aureus ATCC (America Type Culture Collection, ATCC) 25923, Escherichia coli (E. coli) ATCC 25922, Pseudomonas aeruginosa ATCC27853, which were all from the Institute for Combined Injury Lab of 324 Branch Hospital, the People’s Liberation Army of China, had been biochemically identified with sensitivity test results; three clinically isolated fungal strains: Blastomyces albicans, Fusarium solani, Aspergillus fumigatus, were from the Fungal Room, Department of Dermatology, Southwest Hospital, Third Military Medical University, were used.

Preparation of fresh amniotic membrane and fresh amniotic membrane without the epithelium

Fresh amniotic membranes were obtained from healthy parturients under sterile condition using the methods described by Talmi et al. (1991). Any parturients who had used any antibiotics a month before the operation was excluded. Acellular amniotic membranes were prepared with the method as previously described (Meller and Tseng, 1998). Briefly, amniotic membrane was digested for 15 min at 37°C with adding mixture of 0.25% trypsin and 0.06% EDTA, and then the epithelial cells were scraped with a cell scraper. The acellular amniotic membrane was checked using an inverted microscope to make sure that there was no residual epithelial cells or chorion on the amniotic membrane. Lastly, amniotic membranes were fixed with 4% formalin, made into Paraffin sections, stained by HE and observed under light microscope.

Preparation of HAHS

The fresh amniotic membranes with epithelium were rinsed in 0.01 mol / L PBS at 4°C, for 3 times, their wet weights were obtained and then were cut into fragments and homogenated after adding 1:1 of 0.01 mol/L PBS under sterile condition and in ice bath. After these steps, the mixture was broken with an ultrasonator in a 20 ml centrifuge tube and added PBS solution by 1:2. The mixture was then placed in the refrigerated centrifuge (4°C, 2 000 r / min, 10 min), and the supernatant was divided into aseptic package EP tubes and stored at temperature of 4 and -20°C, respectively, until used.

Detection of HAHS’s protein concentration

Bicinchoninic acid (BCA) method was used to determine protein concentration of HAHS. A series of BCA standard products were prepared and standard curve and equation was made. Values of light absorptions of HAHS [D (562)] were then measured. Protein contents were counted according to the standard curve and the equation.

Detection of antibacterial effect of fresh amniotic membrane with epithelium and fresh amniotic membrane without epithelium in vitro, and the antimicrobial activities of fresh HAHS using Kirby-Bauer method were carried out. Ten μl of fluid containing bacteria or fungi was added into a fresh MH agar plate (Streptococcus into Colombia blood plate, fungi into PDA plate). The bacteria (or fungi) were cultured to logarithmic phase and diluted to 5 × 10^2 colony forming unit (CFU)/ml. The bacterial or fungal fluid was evenly coated onto the surface of the medium using a sterilized L-shaped glass bar, and left to dry at room temperature for 2 min. On the surface of the agar, five holes, each with a diameter of 6 mm and equi-distance apart from each other, were made using a hole puncher at room temperature. 50 μl of fresh HAHS at a concentration of 1500 mg/L were added into the periphery 4 holes, while 50 μl of 0.01 mol/LPBS was added to the central hole as negative control. When putting HAHS and PBS into the holes, attention was paid to avoid the HAHS and PBS overflowing of the holes. The bacteria were then cultured for 12 ~ 16 h (fungi for 1 ~ 3 d) at 37°C in a culture incubator.

The anterior (surface with the epithelium rested on) and posterior surface (surface with chorion) of fresh amniotic membrane and a cellular membrane were put onto different agar surface coated with bacterial or fungal fluid without drilling a hole in the membranes.

Comparison of HAHS’s antimicrobial activities with ten antibiotics using Broth Micro-dilution

Method

The ten antibiotics were separately dissolved in ultrapure water at a concentration of 1280 mg / L, and then diluted using an equal volume of sterile 0.02% acetic acid (containing 0.4% BSA). A series of half time diluents (the mass concentration of 640, 320, 160, 80, 40, 20, 10, 5, 2.5, 1.25 mg / L) were obtained by diluting using 0.01% acetic acid (containing 0.2% BSA) solution. The half time diluents were preserved at 4°C until used. Protein content of HAHS was 1500 mg/L prepared in the experiment, and then the protein content was half times diluted by putting 0.01% acetic acid (containing 0.2% BSA) into the diluents to obtain mass concentrations of 1500, 750, 375, 187.5, 93.75, 46.875, 23.4375, 11.719, 5.859, 2.929 mg/L.

Using Staphylococcus aureus ATCC25923 and E. coli ATCC25922 as test pathogens, MIC and MBC of HAHS for each bacterium were compared with five kinds of antibiotics. Minimum inhibitory concentration (MIC) of HAHS for Staphylococcus aureus ATCC25923 was compared with Penicillin, Clindamycin, Chloramphenicol, Rifampicin, and Levofloxacin. MIC of HAHS for E. coli ATCC25922 was compared with Gentamycin, Tobramycin, Sulfacetamide Sodium, Cefuroxime Sodium, and Norfloxacin.

90 μl of bacterial fluid at logarithmic phase at the concentration of 2 × 10^8 ~ 7 × 10^8 CFU / ml were added into the Number 1 to 11 holes of 96 hole plate in order. 100 μl of MH broth were added into the 12th hole as blank control. Ten μl of diluted testing HAHS or antibiotics liquid were separately added into the holes of Number 1 to 10. Ten μl of 0.01 mol / L PBS fluid were added into the 11th hole as negative control. Results were obtained by measuring optical density (D(600)) using 600 nm UV of a ELISA reader after culture in an incubator at 37°C for 12 ~ 16 h. MIC was calculated according to the smallest mass concentration of the hole, which had turbidity less than 50% of the negative control hole (the 11th hole). From two
holes, whose mass concentrations were larger than that of the MIC hole, and no turbidity was observed by naked eye, ten μl of culture medium was sucked out and uniformly coated onto the two MH agar plates separately. CFU were counted after being cultured for 18 h at 37°C. The corresponding mass concentration of the hole, whose CFU number was less than 5 CFU per plate, was considered as the minimal bactericidal concentration (MBC) of HAHS or the antibiotics. The same experiments were conducted separately for three times using the HAHS from three different parturients.

The antibacterial activity of HAHS at different PH values, temperatures and storage time

According to the method comparing antibacterial activity of enzyme dynamics developed by Robinson et al. (1979), the impacts of pH values, temperatures and a range of storage time on antibacterial activity of HAHS were observed by comparing the decreased range of absorbance value D (600) after the testing bacterial fluid was reacted with an equal quantity of HAHS at different level of pH values, temperatures and storage time for 1 h. Staphylococcus aureus ATCC25923 was used as test bacterium.

**pH values**

0.01 mol / L PBS was prepared at five different pH values, pH 5.0, 6.0, 7.0, 8.0, 9.0, and then sterilized by filtration. 25 μl of PBS liquid at the five different pH values was separately blended with five μl. 1 500 mg / L of HAHS in the No. 1 to 5 holes of the 96 hole plate. 30 μl of PBS was added into the 6th hole as negative control. After staying at room temperature for 10 min, 70 μl of 5 × 10⁷ CFU / ml bacterial fluid at logarithmic phase was then added into each hole, and repeated for three times. The D (600) value of the holes was measured immediately after adding the bacterial fluid. The D’ (600) value of each hole was measured after incubation at 37°C for 1 h. △D (600) = D (600) - D’(600) was calculated.

**Temperatures**

EP tubes filled with fresh HAHS were placed at -20, 4, 20, 37, 60 and 80°C, respectively, for 1 h. Five μl of HAHS supernatants at the six different temperatures were mixed with 95 μl of bacterial fluid of 5 × 10⁷ CFU / ml at logarithmic phase in the holes of No. 1 to 6 on the 96 hole-plate. PBS liquid mixed with the bacterial fluid in the 7th hole was served as negative control. The experiment was repeated for three times. D (600) value of the holes were measured immediately after the addition of bacterial fluid, and D’(600) value of the holes were measured after incubation at 37°C for 1 h. △D (600) was calculated.

**Storage time**

Taking 4 and 20°C as storage temperature, respectively, six time points were selected, including 1d, 3d, 7d, 14d, 28d, 56d, to observe the impact of storage time on antibacterial activity of HAHS. The method used was the same as that used for temperatures.

**Observation under transmission electron microscope**

Bacterial fluids of Staphylococcus aureus ATCC 25923 and E. coli ATCC 25922 at logarithmic phase were centrifuged (5000 r / min, 10 min), and the supernatants were disposed. The bacteria were washed using sterile saline and diluted using fresh MHB medium to 10⁸ CFU / ml. One ml of bacterial suspension was taken and 200 μl of 1500 mg / L of HAHS was added in the experimental group and 200 μl saline in the control group. The mixture was centrifuged (2000 r/min, at low temperature) after being placed at 37°C for 30 min. The supernatant was disposed and then the mixture was washed with sterile saline twice, fixed with 2.5% glutaraldehyde, rinsed in 0.01 mol / L PBBS for 10 min, twice, fixed in 1% osmium tetroxide for 2 h, dehydrated in acetone gradient, embedded with 618 epoxy resin, ultra-thinly sliced, polluted with uranium and lead and observed by transmission electron microscope (Netherlands, Philips TECNAI10).

**Statistical analysis**

The experimental data is expressed as X ±SD. One-way ANOVA test was used to compare the difference between groups using statistical software SPSS11.0. And P<0.05 was considered statistically significant.

**RESULTS**

**Antibacterial activities of fresh amniotic membrane with epithelium and fresh amniotic membrane without epithelium in vitro**

Staphylococcus aureus ATCC25923 was used to test the antibacterial effect of fresh amniotic membrane with the epithelium and fresh amniotic membrane without the epithelium. The results showed that the agar with fresh amniotic membrane without the epithelium had no bacteriostatic ring, and the amniotic epithelium was proven to be removed completely by HE staining observed under light microscope. However, there were significant bacteriostatic rings around fresh amniotic membrane with the epithelium, and HE staining also showed that the epithelium was intact.

**Detection of antimicrobial activities of fresh HAHS**

The results indicated that HAHS had marked antibacterial role for the most selected bacteria, including Staphylococcus aureus ATCC25923, E. coli ATCC25922, Pseudomonas aeruginosa ATCC27853, Staphylococcus epidermidis, Proteus mirabilis, S. pneumoniae, Enterococcus faecalis, Fusarium solani, Blastomyces albicans and Aspergillus fumigatus. However, HAHS did not have significant antibacterial effect on clinically isolated multi-drug-resistant Enterobacter cloacae and E. coli and Methicillin-resistant Staphylococcus aureus (MRSA).

**Comparison of MIC and MBC values between HAHS and the 10 kinds of antibiotics**

As antibacterial substances play their antibacterial role through molecular interaction, molality was more suitable
The antibacterial activity of HAHS at different pH values

HAHS had bactericidal effect on *Staphylococcus aureus* ATCC25923 when pH value was 7 (△D (600) = 0.00338). Meanwhile, HAHS had the same bactericidal effect on *Staphylococcus aureus* ATCC25923 at the other four pH values (pH values 5.0, 6.0, 8.0 and 9.0, respectively). The antibacterial activities of HAHS at the five pH values were not significantly different from one another.

**The antibacterial activity of HAHS at different temperatures**

At 37°C, HAHS had bactericidal effect on *Staphylococcus aureus* ATCC25923 (△D (600)=0.00295). When the temperatures were -20, 4, 20 and 60°C, respectively, the bactericidal effect of HAHS had no marked changes from that of 37°C. HAHS at 80°C resulted in its bactericidal action decreasing significantly from that of the other five temperatures (△D (600)=0.00152, P<0.05).

**Impacts of storage time on HAHS antibacterial activity**

We studied the changes of HAHS antibacterial activity when HAHS was stored for 1 d, 3 d, 7d, 14 d, 28 d and 56 d at 4 and 20°C, respectively. The results illustrated that HAHS antibacterial activity did not have significant changes with the extension of storage time at the two temperatures. After storage for 56 d, HAHS could still kill *Staphylococcus aureus* ATCC25923 effectively, △D (600) values of HAHS at 4 and 20°C were 0.00297 and 0.00281, respectively.
Ultrastructural changes of *E. coli* under TEM observation

Cellular walls *E. coli* ATCC25922 showed integrity, smooth edges, and often showed splitting phenomenon. After treatment with HAHS for 1 h, cellular walls of *E. coli* showed shrinkage, the shape of *E. coli* became irregular, the surface was rough and it was difficult to observe splitting phenomenon of *E. coli*. Furthermore, HAHS led to rupture of *E. coli* membrane with releasing of cellular contents.

With *Staphylococcus aureus*, the cellular membrane showed high integrity and active proliferation. After treatment with HAHS for 1 h, the shape of *Staphylococcus aureus* was irregular, the surface was rough and the splitting phenomenon could not be found. In addition, we also observed that the cellular wall of the bacterium was destroyed and protoplast released.

**DISCUSSION**

At present, amniotic membrane stored under deep hypothermia is often used to reconstruct the ocular surface of quiescent disease. The epithelial cells of this amnion have been inactivated. The amnion needs ocular epithelial cells from the recipient to proliferate, migrate, cover and finish epithelization of amnion transplanted area. It was reported that fresh amnion was more effective for reconstructing the ocular surface as reducing inflammatory response, decreasing angiogenesis, and inhibiting proliferation of fibrous tissues (Chen et al., 2000). In our study, fresh amnion with the epithelium and HAHS had significant antibacterial role, while fresh amnion without the epithelium had no antibacterial effect. Our results showed that antibacterial substances of fresh amnion perhaps had something to with the epithelial cells, and need to study.

We also demonstrated that HAHS had a broad antimicrobial spectrum. It had antibacterial effect on *G. staphylococcus* aureus, fungi and even multiple drug-resistant bacteria. In addition, by comparing with the ten antibiotics, antibacterial activity of HAHS was greater than those of Chloramphenicol, Sulfonamides and Cefuroxime, equal to those of Clindamycin and Tobramycin. Moreover, bactericidal activity of HAHS was no less than that of the ten antibiotics. Given that the HAHS we prepared was a mixture, the actual concentration of antibacterial protein should be less than 1500 mg/L. Meanwhile, HAHS was stable at a range of different pH values, temperatures and different storage time. These characteristics make HAHS suitable for clinical application. We considered that HAHS could be an effective and convenient therapy for ocular surface bacterial infectious diseases.

In the present study, the values of MBC and MIC were equal for HAHS. This result illustrated that HAHS’s antibacterial effect was to kill bacteria directly and the TEM results supported this. HAHS we prepared had a greater antibacterial effect on *Staphylococcus aureus* ATCC25923 than *E. coli* ATCC25922. The mechanism needs to be further studied.

**REFERENCES**


