

The Effect of Reduced Bacterial Dilution on Human Amniotic Membrane Antibacterial Activity, in Vitro

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Abstract

Background: The human amniotic membrane is the inner most layer of placenta and has antimicrobial effect, due to the presence of human beta-defensins and elafins. The purpose of this study is to investigate the effect of dilution reduction of 0.5 McFarland prepared from standard bacterial strains of *Salmonella enterica* BAA-708, *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Klebsiella pneumoniae* ATCC7881, and *Enterococcus faecalis* ATCC29212 on antibacterial effect of human amniotic membranes in vitro.

Materials and Methods: The amniotic membranes were obtained from the bank of organ transplantation in Imam Khomeini hospital, of women with elective cesarean section whose HIV, HBV, HCV and VDRL serological tests were negative. They were cut to 1.5×1.5 cm pieces. Then 0.5 McFarland suspensions of 1.5×10^8 , 0.5×10^7 and 1.5×10^6 dilutions were prepared from bacteria which then were spread on Mueller Hinton medium agar and a piece of membrane was put in the center of each plate. After 24 hours incubation at 37°C, the results were observed.

Results: In 0.5 McFarland standard dilution an inhibition zone was created in three standard strains of *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella enterica* unlike the other two strains. There was no change in the above results with two other dilutions and inhibition zone of sensitive strains was not created.

Conclusion: Dilution reduction of microbial strains does not affect the antibacterial impact of amniotic membrane and dilution reduction does not yield to a false positive response and the conversion of resistant to sensitive strains.

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Introduction

Embryonic membrane consists of chorion, allantois, and amnion [1]. Amniotic membrane is the inner most layer of the three constituent layers of the fetal membranes [2]. It is a transparent membrane composed of an inner epithelial layer that is laid on the basement membrane which in turn is connected to a thin membrane of connective tissue through thin filaments comprising of interstitial collagen I, III, and V. The epithelial layer is a single layer of cube-shaped mononuclear cells with some cytoplasmic vacuoles. Basement membrane is thin and contains a fibrous mesh network [3, 4]. Connective tissue is nonvascular mesenchymal tissue [5] and in fact is composed of three layers: compressed layer over basement membrane, fibroblast layer, and spongy layer. The amniotic membrane which thickness is 0.02-0.5 mm covers the amniotic cavity and its apical interior surface is in contact with amniotic fluid while the external surface is in direct contact with the chorionic membrane [3]. Any nerve, muscle or lymph exists in the amnion [5].

Human amniotic membrane has an antibacterial effect [6]. Embryonic membranes and placenta are important sources of natural antimicrobials found in the uterus. The presence of human beta-defensins 1-3 (HBD), elafin, and secretory leukocyte protease inhibitor (SLPI) was shown in the amniotic epithelial layer. HBD-2 is a strong antibiotic and is expressed in response to IL-1 in amniotic epithelial cells [7]. Natural antimicrobials are produced in amniotic fluid during pregnancy and localize in the placenta, uterus endometrium, and fetal membranes [8]. The anti-bacterial effect of amniotic membrane has been shown by Kjaergaard et al. in a broad range of bacteria including *Streptococcus* group A, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* [9]. Given the widespread use of antibiotics, in order to avoid antibiotic resistance, replacement of natural compounds that have antimicrobial properties is inevitable.

To test bacterial sensitivity to antibiotics the disk diffusion method is applied based on NCCLS method that uses a microbial suspension concentration of 0.5

McFarland and antibiotic impregnated disks with a given antibiotic concentration [10]. The purpose of this study is to investigate the effect of dilution reduction of 0.5 McFarland prepared from standard bacterial strains of *Salmonella enterica* BAA-708, *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Klebsiella pneumoniae* ATCC7881, and *Enterococcus faecalis* ATCC29212 on antibacterial effect of human amniotic membranes in vitro.

Materials and Methods

This descriptive cross-sectional study was performed in December 2010 in School of Public Health in Tehran University of Medical Sciences. The human placentas were obtained soon after elective cesarean sections of women whose HIV, HBV, HCV and VDRL serological tests were negative. Then to remove blood clots, they were washed with saline under the laminar flow hood. Inner amniotic membrane was separated from the chorion through blunt dissection. Amniotic membrane was washed three times with phosphate buffer saline (PBS) containing the antibiotics cloxacillin 50 µg/ml, streptomycin 50 µg/ml, and amphotericin B 2.5 µg/ml. In this way, microbial agents during surgery and after delivery were removed. The microbiology tests also took on the membrane [11]. All these steps were performed in the bank of organ transplantation in Imam Khomeini hospital of Tehran city. Membranes were transferred to the laboratory of Tehran University of Medical Sciences in a cold box. Under sterile conditions, under the hood, the membranes were flattened on cellophane so that their epithelial surfaces were upward, then they were washed with sterile saline and were cut into approximately 1.5×1.5 cm pieces by surgical scissors.

In the next step, lyophilized *P. aeruginosa* standard strain (ATCC27853) was transferred from Clinical Microbiology Research Center of Shiraz Medical Sciences University to Tehran and was cultured in BHI medium and incubated for 24 hours at 37°C. *E. faecalis* (ATCC29212), *K. pneumoniae* (ATCC7881), *S. enterica* (BAA-708) and *E. coli* (ATCC25922) strains were obtained from the Microbiology Department of Public Health School of Tehran Medical Sciences University. All 5 strains were cultured on blood agar medium containing

5% sheep blood, then after 24 hours incubation at 37°C, a suspension of 0.5 McFarland was prepared for each strain. In the next step, 1.5×10^7 and 1.5×10^6 dilutions were prepared with 0.5 McFarland suspensions as follows: 1 cc of the 0.5 McFarland suspension of the bacterial strain was dissolved in the 9 cc sterile normal saline and a 1.5×10^7 dilution was prepared from this strain, the same procedure was done for the four remaining bacterial strains. The 1.5×10^6 dilution was prepared as the same procedure from 1.5×10^7 dilution for the 5 standard studied strains.

Then, under sterile conditions and under the hood, 100 µl of prepared dilutions from every 5 standard bacterial strains was poured separately on Mueller Hinton agar medium through lawn method and was uniformly spread over the plate by a sterile swab. Then a piece of the cut amniotic membrane was put in the center of each plate. And finally, 15 cultured plates were incubated for 24 hours at 37°C. In all dilutions, the length of 4 diameters of each inhibition zone was measured by ruler and the mean of diameters were calculated by SPSS-11 software.

Results

The present study which was performed to investigate the impact of dilution reduction on antibacterial effect of human amniotic membrane in vitro, showed the inhibitory effect of amniotic membrane on three standard bacterial strains of *P. aeruginosa*, *E. coli*, and *S. enterica* in 3 studied dilutions, because the inhibition zone was observed in three mentioned strains; while under same conditions, other two strains (*K. pneumoniae* and *E. faecalis*) were resistant to the antibacterial effect of the membrane and the inhibition zone was not observed. The inhibition zone was barely visible in the sensitive strains of *P. aeruginosa* in 3 dilutions.

In each of three studied dilutions (1.5×10^8 , 1.5×10^7 , and 1.5×10^6) no change was observed in the results and diameter of inhibition zone. By reducing the dilution of bacterial suspensions especially from the dilution 1.5×10^7 to 1.5×10^6 , the number of colonies per plate was reduced (Fig. 1).

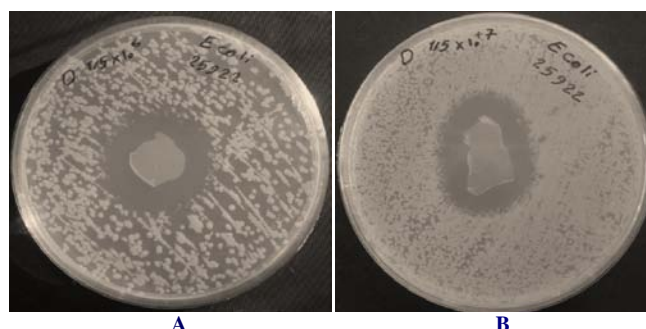


Figure 1. Occurrence of inhibition zone due to the antibacterial effect of amniotic membrane in *E. coli* and the reduction of colony numbers and lack of change in antibacterial effect of membrane in 1.5×10^7 (A) and 1.5×10^6 (B) dilutions

Discussion

The present study revealed the inhibitory effect of amniotic membrane on a specific range of standard bacterial strains including *E. coli* (ATCC25922), *S. enterica* (BAA-708), and *P. aeruginosa* (ATCC27853); however, the two standard strains of *K. pneumoniae* (ATCC7881) and *E. faecalis* (ATCC29212) showed resistance to the antibacterial effect of amniotic membrane.

Kjaergaard et al. examined the antibacterial effect of amniotic and chorionic membranes on strains of *Streptococcus* group A, *Streptococcus* Group B, *S. aureus*, *S. saprophyticus*, *E. faecalis* and reported good results in terms of growth inhibition and inhibition zone diameter in *Streptococcus* Group A, *S. aureus*, and *S. saprophyticus* [12]. Their results confirm our observations regarding to the antibacterial effect of amniotic membrane in creating inhibition zone.

In disk diffusion method based on NCCLS standard, the 0.5 McFarland concentration should be considered, since increase and decrease in dilution level of microbial solution may cause false positive or false negative results [10]. Kjaergaard et al. studied the antibacterial effect of human amniotic membranes on *Streptococcus* group A, *Streptococcus* Group B, *S. aureus*, *S. saprophyticus*, and *E. faecalis* through 0.5 McFarland dilution and disk diffusion methods and observed the inhibition zone in these strains [12]. In the present study because of antibacterial effect of amniotic membrane like an antibiotic, the 0.5 McFarland dilution was used as the standard dilution of the disk diffusion method and in the strains sensitive to the membrane, the inhibition zone was appeared with this dilution. And despite the reduction of suspension dilution of 5 standard strains from 0.5 McFarland (1.5×10^8) to 1.5×10^7

and 1.5×10^6 dilutions, no change was observed in the results obtained from 0.5 McFarland standard dilution and in the inhibition zone diameter in sensitive strains; there was also neither false negative responses nor conversion of resistant to sensitive strains.

Talmi et al. proved the preventing effect of amniotic membrane, chorioamniotic membrane, and polyurethane-based synthetic membranes when grown on agar plates cultured with bacteria. In his research, Talmi used the 3×10^8 and 3×10^6 dilutions of *Streptococcus* Group B microbial suspension [13]. In the present study the microbial suspension was more dilute than Talmi's suspension but the reduction of dilution did not affect the results. Antibacterial effect of human amniotic membrane is stable against various dilutions of the bacterial suspensions; therefore the membrane can be used as a biological material with antibacterial effect along with antibiotics, simultaneously or asynchronously in different bacterial dilutions.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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