Assessment of Antimicrobial Efficacy of Gentamicin-Treated Cryopreserved Amniotic Membrane in an In Vitro Model

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Abstract

Chronic wound infections represent a major global healthcare burden, particularly in patients with diabetes mellitus. Diabetic foot ulcers (DFUs) are frequently complicated by bacterial colonization and biofilm formation, contributing significantly to delayed healing, limb amputation, and increased mortality. In India, the prevalence of diabetic foot complications ranges between 4–10% among diabetic patients, with infection being a leading cause of hospitalization and lower-limb amputation.[1,2] Among the causative organisms, *Pseudomonas aeruginosa* is a clinically important Gram-negative opportunistic pathogen frequently isolated from chronic and burn wounds and is associated with intrinsic resistance mechanisms and biofilm formation.[3]

Introduction

The escalating burden of antimicrobial resistance (AMR), particularly among Gram-negative organisms such as *P. aeruginosa*, poses a substantial therapeutic challenge. The Indian Council of Medical Research (ICMR) antimicrobial resistance surveillance network has reported increasing resistance trends in *Pseudomonas* isolates, underscoring the need for localized antimicrobial strategies and alternative drug-delivery systems.[4] Local antibiotic delivery systems offer the advantage of achieving high drug concentrations at the wound site while minimizing systemic toxicity and resistance pressure.[5]

Human amniotic membrane (HAM), derived from the innermost layer of the placenta, has been extensively investigated for its regenerative and anti-inflammatory properties. It contains extracellular matrix components such as collagen types I, III, IV, V, and VII, fibronectin, laminin, and a variety of growth factors that promote epithelialization and angiogenesis.[6] In addition, HAM exhibits inherent antimicrobial activity attributed to the presence of antimicrobial peptides including defensins, elafin, and secretory leukocyte protease inhibitor (SLPI).[7] However, the intrinsic antimicrobial effect of native HAM may not be sufficient to combat highly virulent or multidrug-resistant organisms.

Previous in-vitro studies have demonstrated that antibiotic-treated amniotic membranes exhibit enhanced antimicrobial activity compared with untreated membranes. Mencucci et al. reported significant inhibition zones against bacterial pathogens when HAM was impregnated with antibiotics, suggesting its potential as a sustained local drug-delivery scaffold.[8] Similarly, Kesting et al. demonstrated antimicrobial effects of HAM against Gram-positive and Gram-negative bacteria, supporting its utility in contaminated wound environments.[9]

Cryopreservation is commonly employed to maintain the structural and biological integrity of HAM for clinical use. Proper cryostorage at –196°C preserves extracellular matrix architecture and growth factors without significantly compromising biological activity.[10] However, limited data exist regarding the stability of antibiotic-loaded cryopreserved HAM and whether antimicrobial efficacy is retained following storage.

Gentamicin, an aminoglycoside antibiotic widely used in the management of Gram-negative infections, including *P. aeruginosa*, acts by inhibiting bacterial protein synthesis through binding to the 30S ribosomal subunit.[11] Its concentration-dependent bactericidal activity makes it an ideal candidate for localized delivery systems.

In this context, the present study aimed to evaluate the antimicrobial activity of gentamicin-treated cryopreserved human amniotic membrane against *Pseudomonas aeruginosa* ATCC 27853 using an in-vitro agar diffusion model. The study further sought to determine whether cryopreservation affects the antimicrobial efficacy of antibiotic-impregnated HAM.

Materials And Methods

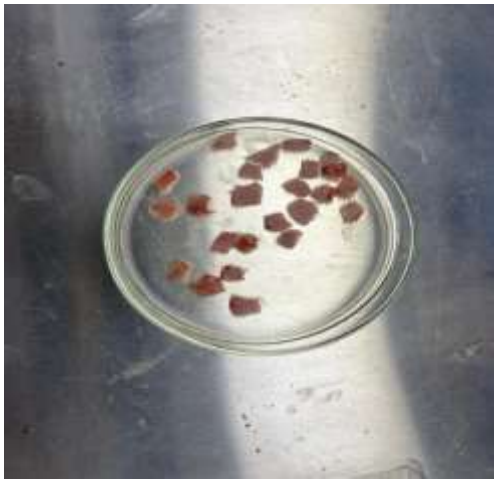
Study Design

This experimental in-vitro study was conducted to evaluate the antimicrobial activity of gentamicin-treated cryopreserved human amniotic membrane (HAM) against *Pseudomonas aeruginosa* ATCC 27853 using an agar diffusion model. The study was designed in accordance with standard microbiological testing principles described by the Clinical and Laboratory Standards Institute (CLSI).[12]

Procurement and Processing of Human Amniotic Membrane

Human placental tissue was obtained from a single healthy donor undergoing elective cesarean section after written informed consent. Donor screening was performed as per institutional protocol to exclude transmissible infections including HIV, hepatitis B virus, hepatitis C virus, and syphilis.

The amniotic membrane was separated from the chorion by blunt dissection under sterile conditions. The membrane was thoroughly rinsed with sterile phosphate-buffered saline (PBS) to remove blood clots and cellular debris. Surface sterilization was performed using ultraviolet irradiation under aseptic conditions. The membrane was then cut into uniform fragments measuring 6 mm × 6 mm using sterile surgical instruments.



Gentamicin Impregnation Protocol

Gentamicin sulfate (analytical grade) solutions were prepared at concentrations of 10 mg/mL, 20 mg/mL, and 40 mg/mL in sterile saline. Untreated membrane fragments served as controls.

For each concentration, six membrane fragments were prepared:

- Three fragments tested prior to cryopreservation (fresh group)
- Three fragments subjected to cryopreservation (cryo group)

Within each subgroup (fresh and cryo), fragments were immersed in gentamicin solution for:

- 5 minutes
- 30 minutes
- 60 minutes

After incubation, each fragment was gently rinsed with sterile saline to remove surface antibiotic residue and blotted on sterile gauze prior to microbiological testing.

Cryopreservation Procedure

For the cryopreservation group, antibiotic-treated membrane fragments were placed in sterile cryomedium (SPERMIFREEZE®, 1:1 ratio) and stored at −196 °C for 7 days.

Prior to testing, cryopreserved membranes were thawed at room temperature and rinsed thoroughly with sterile saline to remove residual cryoprotectant.

Bacterial Strain and Inoculum Preparation

Pseudomonas aeruginosa ATCC 27853 was used as the test organism. This strain is recommended by CLSI as a quality-control strain for antimicrobial susceptibility testing.[12]

A fresh overnight culture was prepared in Mueller–Hinton broth at 37 °C. The bacterial suspension was adjusted to 0.5 McFarland turbidity standard (approximately 1.5×10^8 CFU/mL) using visual comparison with a McFarland standard, as per CLSI guidelines.[12]

Agar Diffusion Assay

Sterile Mueller–Hinton agar plates were uniformly inoculated using a sterile cotton swab dipped in the standardized bacterial suspension to ensure confluent lawn growth.

After allowing the surface to dry for 15 minutes at room temperature, prepared amniotic membrane fragments (fresh and cryopreserved) were aseptically placed onto the agar surface using sterile forceps.

Plates were incubated aerobically at 37 °C for 24 hours.

Measurement of Zone of Inhibition

Following incubation, zones of inhibition were measured in millimeters using a calibrated digital Vernier caliper. Measurements were taken across the maximum diameter of the clear zone surrounding each membrane fragment.

Outcome Measures

The primary outcome was the diameter of the zone of inhibition (mm) produced by each concentration and soaking duration of gentamicin-treated HAM.

Comparative evaluation was performed between:

- Control vs antibiotic-treated groups
- Different concentrations (10, 20, 40 mg/mL)
- Different immersion durations (5, 30, 60 minutes)
- Fresh vs cryopreserved membranes

Statistical Analysis

Due to the experimental design consisting of single membrane samples per concentration and immersion duration, inferential statistical analysis was not performed. Results are presented descriptively as measured inhibition zone diameters (mm). Comparative interpretation was based on observed differences in inhibition zones across concentrations, immersion durations, and between fresh and cryopreserved membranes.

Results

A total of 24 human amniotic membrane (HAM) fragments were evaluated across four concentration groups (control, 10 mg/mL, 20 mg/mL, and 40 mg/mL gentamicin), with assessment performed before and after cryopreservation (7 days at −196 °C).

Antimicrobial Activity of Fresh and Cryopreserved Membranes

The measured zones of inhibition for all experimental conditions are summarised in **Table 1**.

Table 1. Zone of inhibition (mm) of gentamicin-treated human amniotic membrane against *Pseudomonas aeruginosa* ATCC 27853

Group	5 min	30 min	60 min
Fresh – Control	9	10	9
Fresh – 10 mg/mL	28	29	32
Fresh – 20 mg/mL	34	30	30
Fresh – 40 mg/mL	36	32	35
Cryopreserved – Control	9	10	14
Cryopreserved – 10 mg/mL	26	28	30
Cryopreserved – 20 mg/mL	24	32	32
Cryopreserved – 40 mg/mL	40	34	40

Table 1. Zone of inhibition (mm) produced by gentamicin-treated human amniotic membrane against *Pseudomonas aeruginosa* ATCC 27853 at different concentrations and immersion durations. Cryopreserved membranes were stored at −196 °C for 7 days prior to testing.
Fresh gentamicin-treated membranes demonstrated inhibition zones ranging from 28–36 mm across concentrations. The untreated control membranes demonstrated smaller inhibition zones (9–10 mm).
Following cryopreservation, inhibition zones ranged from 24–40 mm. The control in the cryopreserved group measured 9–14 mm.

Concentration-Dependent Pattern
Across both fresh and cryopreserved membranes, increasing gentamicin concentration was generally associated with larger inhibition zones. The 40 mg/mL concentration consistently produced the highest antimicrobial activity in both groups.
This concentration-dependent trend is illustrated in **Figure 1**.

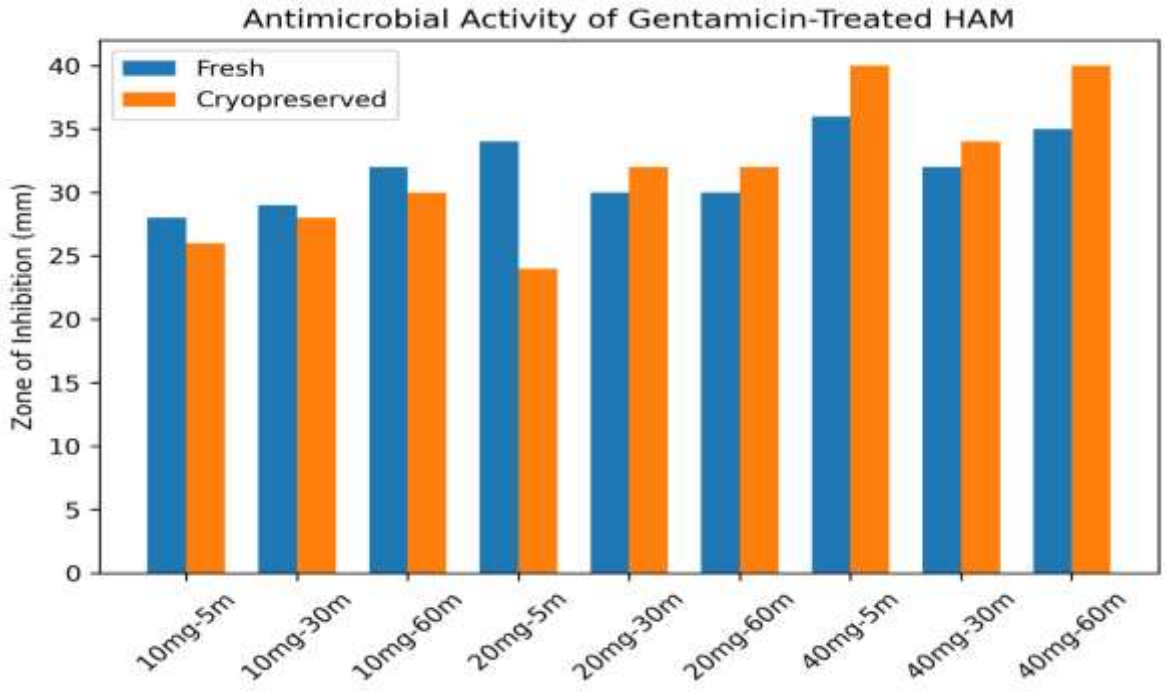
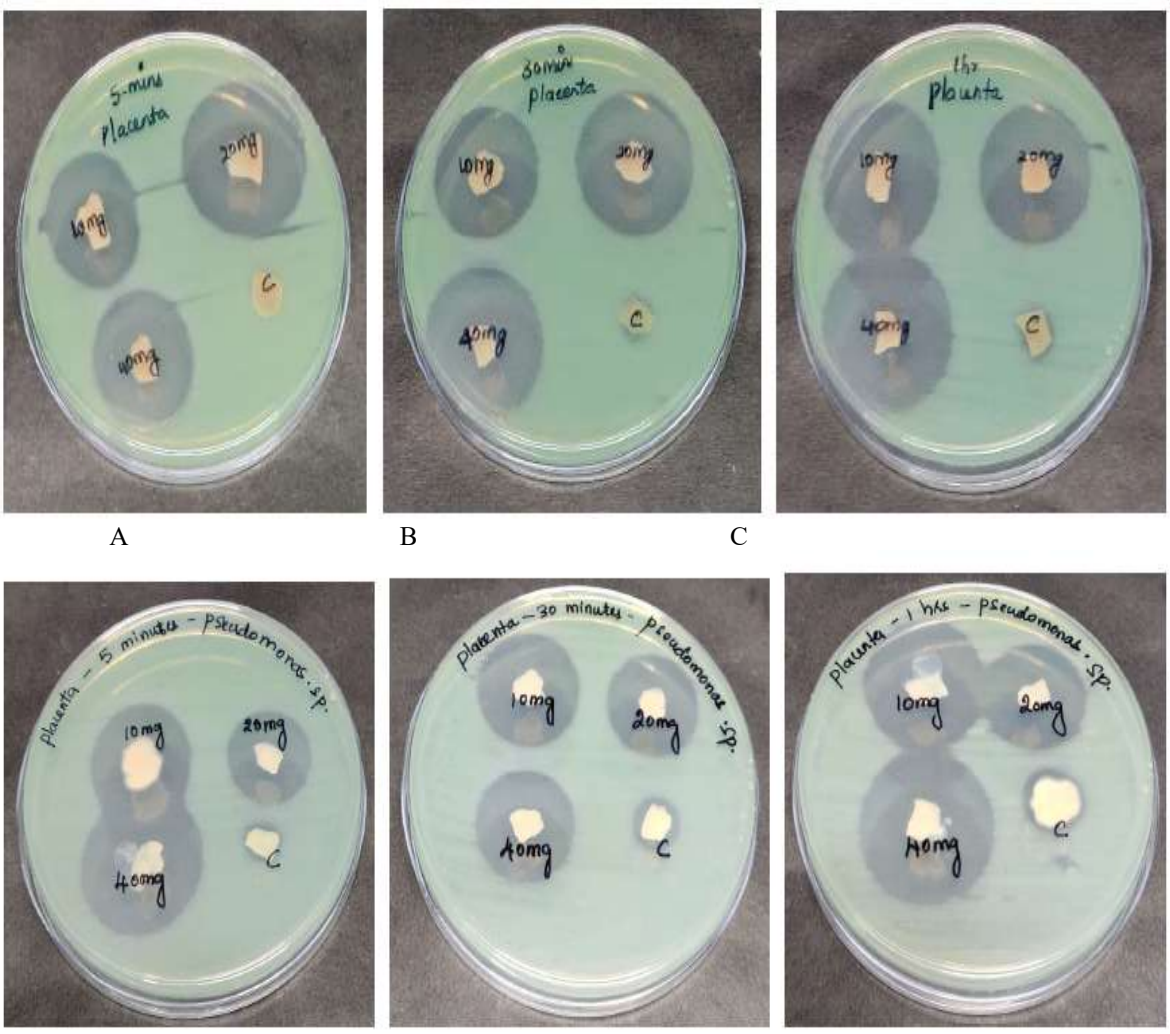


Figure 1. Comparison of antimicrobial activity of fresh and cryopreserved gentamicin-treated human amniotic membrane against *Pseudomonas aeruginosa* ATCC 27853.

Effect of Soaking Duration
At 10 mg/mL concentration, increasing immersion time from 5 minutes to 60 minutes resulted in increased inhibition zone diameter in both fresh (28 → 32 mm) and cryopreserved membranes (26 → 30 mm).
At higher concentrations (20 and 40 mg/mL), the influence of soaking duration was less consistent.

3.4 Effect of Cryopreservation
Cryopreserved membranes retained antimicrobial activity across all concentrations. In selected conditions (40 mg/mL at 5 and 60 minutes), cryopreserved membranes demonstrated inhibition zones equal to or greater than those of fresh membranes.

Given the pilot nature of the study and single-fragment testing per condition, findings are presented descriptively without inferential statistical testing.



A B C

D E F

Figure 2. Representative agar diffusion plates demonstrating antimicrobial activity of gentamicin-treated human amniotic membrane against *Pseudomonas aeruginosa* ATCC 27853.

(A–C) Fresh membranes were immersed for 5, 30, and 60 minutes, respectively.
(D–F) Cryopreserved membranes (7 days at −196 °C) were immersed for 5, 30, and 60minutes.
Each plate contains control (C) and gentamicin-treated membrane fragments (10, 20, and 40 mg/mL).

Discussion

The present pilot in-vitro experimental study evaluated the antimicrobial activity of gentamicin-treated cryopreserved human amniotic membrane (HAM) against *Pseudomonas aeruginosa* ATCC 27853. The findings demonstrate that antibiotic-impregnated HAM exhibits measurable antimicrobial activity across all tested concentrations, and that this activity is retained following cryopreservation.

Gentamicin-Loaded HAM as a Local Drug-Delivery Matrix

Local antimicrobial delivery systems are increasingly explored as adjuncts in the management of infected wounds, particularly in the context of antimicrobial resistance. Localized delivery allows high drug concentration at the site of infection while minimizing systemic toxicity and resistance selection pressure.[13] Collagen-based scaffolds and biological matrices have been investigated as carriers for antibiotics due to their biocompatibility and sustained release properties.[14]

The human amniotic membrane is composed predominantly of collagen types I, III, IV, V, and VII, along with glycoproteins and proteoglycans, which collectively provide a semi-permeable extracellular matrix capable of drug absorption and gradual release.[6] The observed concentration-dependent increase in inhibition zones in the present study supports the concept that HAM can function as a passive antibiotic reservoir.

Similar findings were reported by Mencucci et al., who demonstrated enhanced inhibition zones when HAM was treated with topical antibiotics in an in-vitro model.[8] The magnitude of inhibition observed in the present study (up to 40 mm) is consistent with previously reported antimicrobial enhancement following antibiotic impregnation of biological membranes.[9]

Dose-Dependent Antimicrobial Activity

Across both fresh and cryopreserved groups, higher gentamicin concentrations generally produced larger inhibition zones. Gentamicin exhibits concentration-dependent bactericidal activity against Gram-negative organisms, including *P. aeruginosa*, mediated through irreversible binding to the 30S ribosomal subunit and disruption of protein synthesis.[11]

The increase in inhibition diameter at 40 mg/mL likely reflects greater antibiotic uptake and local diffusion from the membrane matrix. Concentration-dependent antimicrobial response has been previously demonstrated in aminoglycoside-based delivery systems.[15]

Influence of Soaking Duration

In the 10 mg/mL group, increased soaking duration was associated with larger inhibition zones, suggesting time-dependent absorption of gentamicin into the membrane matrix. However, this effect was less pronounced at higher concentrations, possibly indicating early saturation of binding sites within the collagen matrix.

The kinetics of antibiotic uptake in biological scaffolds has been shown to reach equilibrium rapidly under higher concentration gradients.[16] This may explain the modest incremental benefit of prolonged immersion observed at 20 and 40 mg/mL.

Retention of Antimicrobial Activity After Cryopreservation

A key objective of the study was to assess whether cryopreservation alters antimicrobial efficacy. Cryopreserved membranes retained inhibition zones comparable to fresh membranes, suggesting preservation of antibiotic-binding capacity and matrix integrity.

Previous studies evaluating cryopreserved amniotic membrane have demonstrated maintenance of structural collagen architecture and biological properties following storage at −196 °C.[10] Furthermore, preserved extracellular matrix integrity is essential for sustained drug retention and release.

The present findings indicate that short-term cryopreservation (7 days) does not compromise the antimicrobial potential of gentamicin-loaded HAM. This has important implications for clinical storage and staged application.

Intrinsic Antimicrobial Activity of Untreated HAM

The untreated control membranes demonstrated smaller but measurable zones of inhibition (9–14 mm). Human amniotic membrane is known to contain antimicrobial peptides such as β-defensins, elafin, and secretory leukocyte protease inhibitor, which may account for this intrinsic inhibitory effect.[7,17]

Kjaergaard et al. previously reported antibacterial properties of amnion and chorion in vitro, supporting the concept that native HAM possesses baseline antimicrobial activity.[18] The present findings are consistent with those observations.

Clinical Implications

Pseudomonas aeruginosa remains a major pathogen in chronic wound infections, particularly in diabetic foot ulcers and burn wounds.[3] The ability of gentamicin-loaded HAM to generate substantial inhibition zones suggests potential utility as a localised adjunct in managing contaminated or infected wounds.

In India, rising antimicrobial resistance among Gram-negative pathogens has been documented through national surveillance programs.[4] Biological drug-delivery matrices such as HAM may provide a cost-effective, locally applicable alternative strategy in resource-limited settings.

Study Limitations

This study has several limitations. First, it was conducted as a pilot in-vitro experiment using a single donor membrane source. Second, each condition was represented by a single membrane fragment, precluding inferential statistical analysis. Third, only one bacterial strain (*P. aeruginosa* ATCC 27853) was evaluated.

Future investigations should incorporate multi-donor sampling, technical replicates, extended storage duration, and in-vivo validation in infected wound models.

Conclusion

Within the limitations of this pilot in-vitro study, gentamicin-treated cryopreserved human amniotic membrane demonstrated concentration-dependent antimicrobial activity against *Pseudomonas aeruginosa*, with retention of efficacy following cryopreservation. These findings support further exploration of HAM as a biological antibiotic-delivery scaffold in infected wound management.

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