In Vitro Cytocompatibility Assessment of Gentamicin-Impregnated Cryopreserved Amniotic Membrane Using 3T3-L1 Fibroblast Cell Line

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Abstract

Background: Human amniotic membrane (HAM) has emerged as a promising biological scaffold in regenerative medicine owing to its anti-inflammatory, antimicrobial, and wound healing properties. Antibiotic impregnation of cryopreserved amniotic membrane may further enhance its therapeutic utility in infected chronic wounds such as diabetic foot ulcers. However, evaluation of its cytocompatibility is essential before clinical application.

Aim: To evaluate the in vitro cytocompatibility and morphological effects of gentamicin-impregnated cryopreserved amniotic membrane on mouse fibroblast cell line (3T3-L1) using MTT assay and phase-contrast microscopy.

Materials and Methods: Mouse fibroblast cell line (3T3-L1) was cultured in RPMI medium supplemented with 10% fetal bovine serum and antibiotics under standard conditions. Cells were exposed to gentamicin-impregnated cryopreserved amniotic membrane preparations containing three different gentamicin concentrations (40, 80, and 120 µg/mL), designated as 40 AM 276, 80 AM 125, and 120 AM 166, respectively. Cell viability was assessed at 24, 48, and 72 hours using the MTT assay. Morphological alterations were evaluated using inverted phase-contrast microscopy. Statistical analysis was performed using one-way ANOVA, and $p<0.05$ was considered statistically significant.

Results: All experimental groups demonstrated high fibroblast viability throughout the study duration. Mean cell viability remained above 90% in all groups at 24, 48, and 72 hours, indicating no significant cytotoxicity. The 40 AM 276 group demonstrated significantly higher viability at 48 and 72 hours compared with other groups ($p<0.05$). Morphological evaluation revealed preserved fibroblast architecture with spindle-shaped morphology and intact cellular adherence across all groups without evidence of cellular degeneration or membrane damage.

Conclusion: Gentamicin-impregnated cryopreserved amniotic membrane demonstrated excellent in vitro cytocompatibility with preserved fibroblast viability and morphology. These findings support its potential utility as a biologically compatible wound dressing material for regenerative wound management applications.

Keywords: Amniotic membrane; Cryopreservation; Gentamicin; Fibroblast; MTT assay; Cytocompatibility; Tissue engineering; Wound healing; 3T3-L1; Biomaterial.

Introduction

Chronic wounds continue to represent a major global healthcare challenge, particularly among patients with diabetes mellitus, vascular insufficiency, and immunocompromised states. Delayed wound healing contributes substantially to morbidity, prolonged hospitalization, repeated surgical interventions, and increased healthcare expenditure. Among chronic wounds, diabetic foot ulcers remain one of the leading causes of non-traumatic lower limb amputations worldwide.[1]

Successful wound healing requires a coordinated interaction between inflammatory mediators, extracellular matrix remodeling, angiogenesis, fibroblast proliferation, and epithelial regeneration. In chronic wounds, this process becomes disrupted due to persistent inflammation, impaired angiogenesis, oxidative stress, and microbial colonization.[2] Fibroblasts play a crucial role in wound repair through collagen synthesis, extracellular matrix deposition, and growth factor secretion. Therefore, preservation of fibroblast viability is considered an essential indicator of biomaterial biocompatibility.[3]

Human amniotic membrane (HAM), derived from the innermost layer of the placenta, has gained significant attention in regenerative medicine because of its unique biological properties. HAM contains collagen, laminin, fibronectin, growth factors, and cytokines that promote epithelialization, angiogenesis, and tissue repair.[4] Additionally, HAM demonstrates anti-inflammatory, anti-fibrotic, anti-scarring, and antimicrobial properties with minimal immunogenicity.[5]

Cryopreservation of amniotic membrane enables long-term storage while preserving structural integrity and biological activity.[6] Furthermore, incorporation of antimicrobial agents into biological scaffolds has emerged as an attractive strategy for simultaneous infection control and tissue regeneration. Gentamicin is a broad-spectrum aminoglycoside antibiotic widely used against gram-negative wound pathogens and has demonstrated favorable efficacy in topical wound management.[7]

Although several studies have evaluated the regenerative potential of amniotic membrane in chronic wound healing, data regarding the cytocompatibility of antibiotic-impregnated cryopreserved amniotic membrane remain limited. Before clinical application, it is essential to determine whether antibiotic loading affects cellular viability and morphology. Hence, the present study was undertaken to evaluate the in vitro cytocompatibility and

morphological effects of gentamicin-impregnated cryopreserved amniotic membrane using 3T3-L1 fibroblast cell line.

Materials and Methods

Study Design

This was an in vitro experimental laboratory study conducted to evaluate the cytocompatibility of gentamicin-impregnated cryopreserved amniotic membrane using a mouse fibroblast cell line (3T3-L1).

Cell Line Maintenance

Mouse fibroblast cell line (3T3-L1) was obtained from the National Centre for Cell Science (NCCS), Pune. Cells were cultured in T25 culture flasks containing RPMI medium supplemented with 10% fetal bovine serum and 1% antibiotic solution. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Upon reaching confluency, cells were trypsinized and passaged for further experiments.

Preparation of Experimental Groups

Gentamicin-impregnated cryopreserved amniotic membrane samples were categorized into three experimental groups based on antibiotic concentration:

- Group I – 40 AM 276
- Group II – 80 AM 125
- Group III – 120 AM 166

Untreated fibroblast cells served as the control group.

MTT Cell Viability Assay

The fibroblast cell line (3T3-L1) was seeded into 96-well plates at a concentration of 5×10^3 cells/well in RPMI medium supplemented with fetal bovine serum and antibiotics. Cells were incubated at 37°C with 5% CO_2. After washing with phosphate-buffered saline (PBS), cells were exposed to gentamicin-impregnated cryopreserved amniotic membrane samples containing varying gentamicin concentrations (40, 80, and 120 µg/mL), designated as 40 AM 276, 80 AM 125, and 120 AM 166, respectively, and incubated for 24, 48, and 72 hours.

At the end of each incubation period, the medium was aspirated and 0.5 mg/mL MTT solution prepared in PBS was added to each well and incubated for 4 hours. The resulting formazan crystals were dissolved using dimethyl sulfoxide (DMSO), and absorbance was measured at 570 nm using a microplate reader.

Cell viability was calculated using the formula:

$$\text{Cell Viability (\%)} = \frac{\text{OD of treated cells}}{\text{OD of control cells}} \times 100$$

Morphological Analysis

For morphological evaluation, 3T3-L1 fibroblast cells were seeded in 6-well plates at a density of 2×10^5 cells/well. Cells were treated with the experimental membrane preparations and incubated for 24, 48, and 72 hours. After incubation, cells were washed with PBS and examined using an inverted phase-contrast microscope to assess cellular morphology and adherence.

Statistical Analysis

Statistical analysis was performed using SPSS software version 26.0 (IBM Corp., Armonk, NY, USA). Data were represented as mean ± standard deviation (SD) of triplicate experiments. One-way ANOVA was used for intergroup comparisons. A p-value <0.05 was considered statistically significant.

Results

Cell Viability Analysis

The MTT assay demonstrated high fibroblast viability in all experimental groups at 24, 48, and 72 hours, indicating no significant cytotoxicity of gentamicin-impregnated cryopreserved amniotic membrane.

At 24 hours, the mean percentage viability ranged from 96.17% to 99.91% among experimental groups. The 40 AM 276 group demonstrated significantly higher viability at 48 hours ($p = 0.03$) and 72 hours ($p = 0.01$) compared with other experimental groups.

At 48 hours, the 40 AM 276 group showed significantly increased cell viability (99.72%) compared with 80 AM 125 (97.27%) and 120 AM 166 (95.12%). Statistical significance was observed between experimental groups ($p<0.05$).

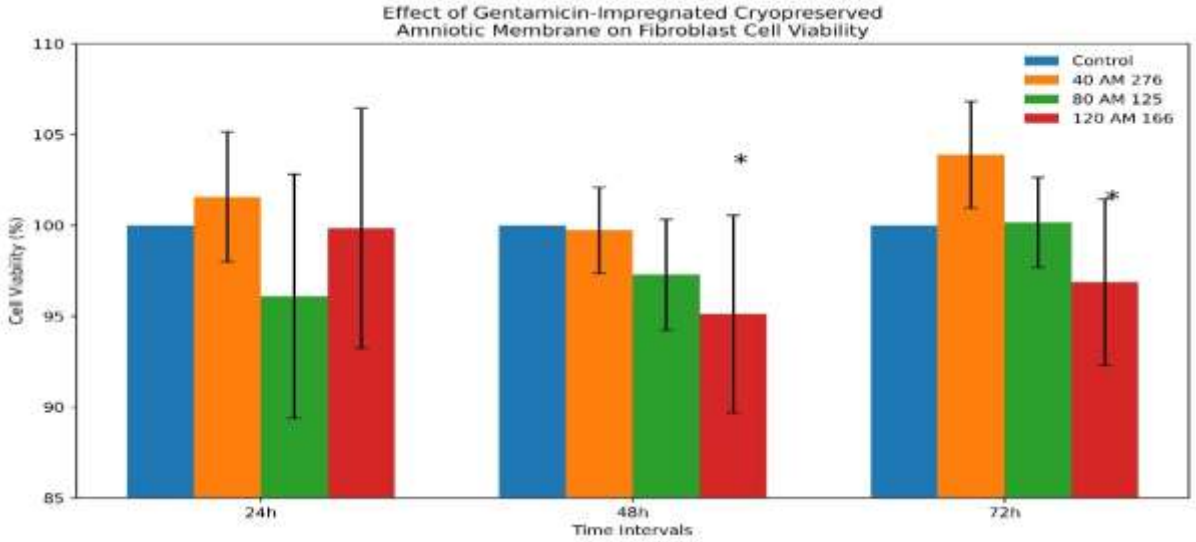
At 72 hours, fibroblast viability remained preserved in all groups. The 40 AM 276 group maintained the highest viability, while the 120 AM 166 group demonstrated a mild reduction in viability compared with controls; however, overall viability remained above 90%.

These findings suggest that gentamicin-impregnated cryopreserved amniotic membrane possesses excellent cytocompatibility with preserved fibroblast metabolic activity.

Table 1. Percentage Cell Viability at Different Time Intervals

Time Point	Control	40 AM 276	80 AM 125	120 AM 166	p-value
24 hours	100	101.56 ± 3.58	96.10 ± 6.71	99.84 ± 6.61	0.45
48 hours	100	99.72 ± 2.37	97.27 ± 3.05	95.12 ± 5.43	0.03
72 hours	100	103.89 ± 2.94	100.16 ± 2.48	96.87 ± 4.58	0.01

Figure 1. Effect of Gentamicin-Impregnated Cryopreserved Amniotic Membrane on Fibroblast Cell Viability.



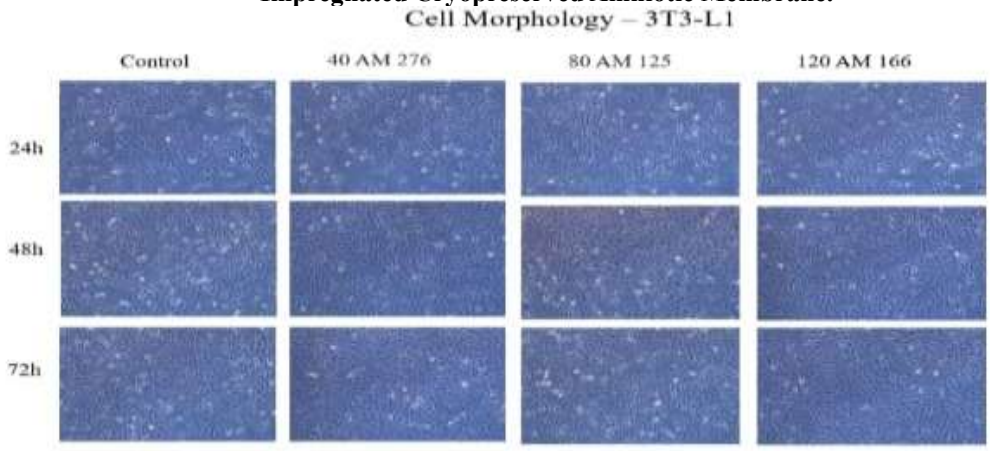
Bar graph demonstrating percentage viability of 3T3-L1 fibroblast cells following exposure to gentamicin-impregnated cryopreserved amniotic membrane preparations at 24, 48, and 72 hours. Data are represented as mean ± SD of triplicate experiments. *$p < 0.05$ between experimental groups

Morphological Evaluation

Microscopic examination revealed preserved fibroblast morphology across all experimental groups at 24, 48, and 72 hours. Cells exhibited normal spindle-shaped fibroblastic architecture with intact cellular adherence and confluent growth patterns.

No significant morphological evidence of cytotoxicity such as cell shrinkage, membrane blebbing, vacuolization, or cellular detachment was observed. The findings indicate that gentamicin impregnation did not adversely affect fibroblast structural integrity.

Figure 2. Morphological Assessment of 3T3-L1 Fibroblast Cells Following Exposure to Gentamicin-Impregnated Cryopreserved Amniotic Membrane.



Representative phase-contrast microscopic images demonstrating preserved spindle-shaped fibroblast morphology and cellular adherence across all experimental groups at 24, 48, and 72 hours without evidence of significant cytotoxic alterations.

Discussion

The present study evaluated the cytocompatibility of gentamicin-impregnated cryopreserved amniotic membrane using the 3T3-L1 fibroblast cell line. The findings demonstrated preserved fibroblast viability and morphology across all experimental groups, indicating that antibiotic impregnation did not induce significant cytotoxic effects. Fibroblast viability is a critical determinant in evaluating the safety of biomaterials intended for wound healing applications. Fibroblasts are essential for extracellular matrix deposition, collagen synthesis, and tissue

remodelling during wound repair.[8] Therefore, preservation of fibroblast function is indicative of favourable biomaterial biocompatibility.

The preserved fibroblast morphology observed in the present study further supports the biological compatibility of gentamicin-impregnated cryopreserved amniotic membrane. Fibroblast adherence, spindle-shaped architecture, and absence of cellular degeneration indicate maintenance of cellular integrity following membrane exposure. Similar findings have been reported in previous studies evaluating amniotic membrane-based biomaterials for regenerative wound applications.

Human amniotic membrane has been extensively investigated as a biological scaffold because of its extracellular matrix composition, growth factor content, and anti-inflammatory properties.[4] Niknejad et al. reported that amniotic membrane promotes epithelial cell migration and fibroblast proliferation owing to its collagen-rich matrix and growth factor reservoir.[4] Similar findings were observed in the present study, where fibroblast morphology remained preserved throughout the experimental duration.

The present study also demonstrated that gentamicin impregnation did not compromise fibroblast metabolic activity. This finding is clinically relevant because chronic wounds frequently harbor polymicrobial infections requiring antimicrobial intervention. Localized antibiotic delivery through biological scaffolds may provide sustained antimicrobial activity while minimizing systemic toxicity.[9]

Previous studies have demonstrated the regenerative potential of cryopreserved amniotic membrane in chronic wound healing. Serena et al. observed improved wound closure rates in diabetic foot ulcers treated with amniotic membrane compared with conventional therapy.[10] Additionally, Mencucci et al. demonstrated enhanced antimicrobial activity of antibiotic-treated amniotic membrane without significant alteration in membrane integrity.[11] The current findings support these observations by confirming favorable cytocompatibility following gentamicin impregnation.

Morphological analysis further reinforced the biocompatibility profile of the membrane preparations. Cells maintained normal spindle-shaped morphology with preserved adherence and absence of degenerative changes. Similar morphological preservation has been reported in previous cytocompatibility studies involving biological scaffolds and amniotic membrane derivatives.[12]

The present study possesses certain limitations. The study was conducted under in vitro conditions using a single fibroblast cell line and did not evaluate long-term cellular interactions or antimicrobial efficacy against wound pathogens. Additionally, quantitative inflammatory markers and growth factor analysis were not performed. Nevertheless, the findings provide important preliminary evidence supporting the cytocompatibility of gentamicin-impregnated cryopreserved amniotic membrane.

Future studies involving animal wound models and clinical trials are necessary to establish its therapeutic efficacy in chronic wound management.

Conclusion

Gentamicin-impregnated cryopreserved amniotic membrane demonstrated excellent in vitro cytocompatibility with preserved fibroblast viability and morphology across all experimental groups. The membrane exhibited minimal cytotoxicity and maintained cellular structural integrity over 72 hours. These findings support its potential as a biologically compatible wound-dressing material for regenerative wound management and chronic wound therapy.

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