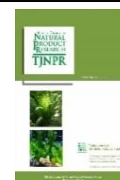
Trop J Nat Prod Res, February 2025; 9(2): 642 – 645 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article



Evaluation of Osteoblast and Fibroblast Viability on Bovine Amniotic Membrane

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ARTICLE INFO

Article history:
Received 10 December 2024
Revised 20 December 2024
Accepted 13 February 2025
Published online 01 March 2025

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ABSTRACT

Guided tissue regeneration (GTR), as one of the periodontal treatments, is a reconstructive periodontal surgical technique where scaffold is an important element. Bovine amniotic membrane (BAM) has anti-inflammatory and antibacterial properties, also multiple growth factors which are useful for periodontal healing and regeneration processes. As a biomaterial with GTR potential, BAM must meet the criteria for membrane barriers, including biocompatibility. This study aimed to determine the viability of osteoblast and fibroblast cells following the application of bovine amnion membranes. The study used a design featuring a laboratory experiment with a post-test-only control group. The cells were divided into five groups, each with seven replicates. Osteoblast and fibroblast cells were treated with BAM for 24 hours, after which the viability of the osteoblast and fibroblast cells was determined using the microculture tetrazolium assay. Consequently, after the intervention, osteoblast and fibroblast cells vitality was 91.73% and 98.4%, respectively. The statistical analysis indicated no significant difference between the cell control and treatment groups. This finding demonstrates that post-administration of BAM, osteoblast and fibroblast cells displayed elevated cell viability, with a live cell percentage above 50%. This indicated that BAM is safe and non-toxic to osteoblast and fibroblast cells.

Keywords: Bovine amniotic membrane, Osteoblast, Fibroblast, Viability, Microculture Tetrazolium assay.

Introduction

Amniotic membranes have been utilized in medicine for decades as a biodegradable substance.[1] In the medical domain, the human amniotic membrane (HAM) is recognized for its capacity to expedite the healing of epithelial injuries, such as burns, chronic ulcers, and conjunctival injuries. Nonetheless, the use of HAM in Indonesia is constrained by legal and religious concerns and its accessibility.[2] The potential transmission of bacterial, viral, or fungal infections from the donor, if not meticulously evaluated, constitutes a vulnerability of the HAM.[3] The bovine amniotic membrane (BAM) is readily available, allowing for large scale production and serving as an alternative to HAM.[4] The BAM, the innermost layer of the fetal placenta abundant in collagen and various growth factors that enhance re-epithelialization in wound healing, has garnered extensive research interest and application as a guided tissue regeneration method in periodontal therapy due to its antibacterial and anti-inflammatory properties.[4-7] Fresh membranes, desiccated membranes, and lyophilized membranes represent various kinds of BAM. The freeze-dried amniotic membrane possesses a sponge-like porous structure.[4]

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Citation: Ulfah N, Setiawatie EM, Bargowo L, Supandi SK, Larasati DM, Fitrina EY, Putra IGAA, Sekartaji DKD, Sayidinar KR. Evaluation of Osteoblast and Fibroblast Viability on Bovine Amniotic Membrane Trop J Nat Prod Res. 2025; 9(2): 642 – 645 https://doi.org/10.26538/tjnpr/v9i2.28

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There has been limited research on bovine amnion membranes. Membranes are frequently used with bone transplants to facilitate and enhance bone regeneration by affecting various bone cells, such as osteoblasts.[8,9] In addition to osteoblasts, fibroblasts are fundamentally incorporated within the composition of contemporary periodontal tissue structures during the healing response. Fibroblasts synthesize and arrange the collagen fibres that connect the cementum of the tooth roots to the gingiva and alveolar bone.[10]
The bovine amnionic membrane should not adversely affect osteoblast and fibroblast cells to ensure the proper functioning of bone and tissue repair processes. As a biomaterial intended for direct bodily interaction, it must possess biocompatible and non-toxic characteristics.[11] A viability test is one of the *in vitro* assessments used to ascertain cytotoxicity. Currently, research on the application of BAM in periodontal therapy is limited. This study aimed to determine the viability of fibroblast and osteoblast cell cultures after BAM administration. The findings from this study are expected to provide evidence for further research, paving the way for the use of BAM as an alternative biomaterial in periodontal tissue treatment.

Materials and Methods

Materials
The bovine amniotic membrane used in this study is the Freeze-dried bovine amniotic membrane (BATAN® Amnion Membrane, Batan Research Tissue Bank). Baby Hamster Kidney (BHK)-21 fibroblast cells and osteoblast cells isolated from rat calvaria.

Ethical clearance
Ethical clearance with reference number 1204/HRECC.FODM/X/2023 was obtained from the Central Laboratory of Veterinary Farma Surabaya, Indonesia.[12,13]

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ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

figCulture of osteoblast cells
Osteoblast cell cultures derived from the calvaria of 2-day-old rats were placed in Roux bottles (DURAN® Culture Flask, Roux Type, Germany)· containing Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS) (PAN Biotech GmbH, Aidenbach, Germany), which appeared uniformly distributed under a light microscope (Models IS.1153-EPL, Euromex iScope Microscope Material Science Trinocular IS1053PLMi, Mexico). The cell culture was incubated in a typical CO_2 incubator (NuAire DHD AutoFlow Model 5510g Air Jacketed CO_2 Incubator, California) at 37°C for 24 hours. After rinsing with 3-5 mL of phosphate-buffered saline (PBS), the osteoblast cell culture was detached using 0.5% trypsin-versene for 20 minutes and subsequently resuspended in a growth medium containing 10% fetal bovine serum (FBS). Growth media (10 µL) and cell culture were incubated at 37°C for 24 hours in a 5% CO2 environment.[14,15]

Culture of fibroblast cell
Fifty microliters of Baby Hamster Kidney (BHK)-21 fibroblast cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) in Roux culture bottles and subsequently plated in each well of a 96-well microplate at a density of 2.4 x 10^4 cells/mL, followed by incubation at 37°C for 48 hours.[12]

Treatment with BAM and MTT Assay
Osteoblast cell culture and BHK-21 fibroblast cells (50 µL) in a 96-well microplate were administered 0.1 mg of freeze-dried bovine amniotic membrane. The cells were reproduced seven times and cultured in a CO_2 incubator at 37°C for 24 hours.[12] After removing the media, the wells were rinsed twice with 200 µL of phosphate-buffered saline (PBS). Each well received 40 µL of fresh culture medium and 10 µL of MTT [3-(4,5-Dimethylthiazol-2-yl) 2,5-Diphenyltetrazolium Bromide] reagent (5 mg/mL), followed by a 4-hour incubation in a CO_2 incubator at 37°C. The medium from each well was extracted with a syringe, followed by addition of 50 µL of dimethyl sulfoxide (DMSO), and the mixture was agitated using a plate shaker for 5 minutes until the formazan crystals were fully dissolved.[14,15]
The absorbance of the formazan was measured spectrophotometrically using an ELISA reader (Multiskan™ FC Microplate Photometer, Indonesia) at a wavelength of 570 nm.[12] The absorbance value and the number of viable fibroblast or osteoblast cells exhibit a positive correlation with the intensity of the colour.[11,14] Following the MTT assay, the fibroblasts and osteoblasts for both control and intervention groups were observed under a light microscope.[16] Viable fibroblast cells were purplish-blue when stained with formazan (Figure 1a). Viable osteoblast cells retain a blue colour in the presence of formazan (Figure 1c). The viability of living cells was determined using the equation for cell viability and the toxicity parameter based on CD_{50} (Cytotoxic Dose). The intervention is considered non-toxic if the cell viability exceeds 50% (>50%).[16-18] The proportions of viable fibroblast and osteoblast cells was determined using the following equations:

$$Viable\ fibroblast\ cells(\%) = \frac{OD\ BAM\ fibroblast - OD\ control\ media}{OD\ control\ cell\ fibroblast - OD\ control\ media} x100\%$$

Where;
OD = Optical density,
BAM fibroblast = 50 µL DMEM + 50 µL BHK-21 fibroblast cells + freeze-dried bovine amniotic membrane (0.1 mg),
Control cell fibroblast = 50 µL DMEM + BHK-21 fibroblast cells,
Control media = 50 µL DMEM

$$Viable\ osteoblast\ cells\ (\%) = \frac{OD\ BAM\ osteoblast - OD\ control\ media}{OD\ control\ cell\ osteoblast - OD\ control\ media} x100\%$$

Where;
OD = Optical density,
BAM osteoblast = 50 µL DMEM + 10 µL osteoblast cell culture + freeze-dried bovine amniotic membrane (0.1 mg),
Control cell osteoblast = 50 µL DMEM + 10 µL osteoblast cell culture,
Control media = 50 µL DMEM

Statistical analysis
Data were analyzed by One Way analysis of variance (ANOVA) using the statistical package for the social sciences (SPSS IBM 25 Software, 2017). The Least Significant Difference (LSD) test was used to assess the differences between the intervention and control groups. In each analysis, P-value less than 0.05 was considered statistically significant.[15,19]

Results and Discussion

The MTT colorimetric assay was used in this study to assess the viability of the fibroblast and osteoblast cells treated with bovine amniotic membrane (BAM). In this colorimetric method, the absorbance values are indicative of the number of viable cells and their metabolic activity.[20,21] The quantity of formazan crystal formed serves as the basis for the test and a positive correlation with cell quantity and metabolic activity. The MTT assay is rapid, precise, and appropriate for extended testing.[13,21] For medical treatment biomaterials, cell viability assessment is essential for meeting biocompatibility standards. A recent investigation by Suroto *et al*. (2024) indicated BAM cytocompatibility, with cell viability surpassing 70%, it was suggested that BAM may be an alternative to HAM.[22] The present investigation was done to ascertain that BAM is non-toxic and does not induce cell death in osteoblasts and fibroblasts, as these cells are essential for bone remodelling and wound healing.

Effect of BAM on fibroblast cell viability
Fibroblasts synthesize regulatory substances and engage with other cells involved in the healing process to regulate the complete repair mechanism.[23] The absorbance of the purple formazan crystals produced from the reaction of the MTT reagent with the mitochondrial enzymes of metabolically active cells, was used to assess cell viability. Enzymes are located in the mitochondria of living, metabolically active cells.[12] The succinate dehydrogenase enzyme is transformed into purplish-blue formazan crystals by living cells, observable under a light microscope following a 24-hour MTT assay for the fibroblast control cell group (Figure 1a) and the BAM group (Figure 1b). The quantity of viable cells is directly proportional to the intensity of the purple colour. The percentage cell viability exceeded 50%. The study demonstrated a 98.44% efficacy, indicating that BAM exerts a non-toxic effect on BHK-21 fibroblast cells. This is in agreement with the study of Octarina *et al*. (2022) which reported a fibroblast cell viability of 98.14% after treatment with BAM.[24]
The data obtained were subjected to normality assessment using the Shapiro-Wilk test, and this revealed a normal distribution ($p > 0.216$). The data were assessed for homogeneity using Levene's tests to evaluate the uniformity of fibroblast cells. The test resulted in a p-value of 0.088 ($p > 0.05$), suggesting that the data collected was homogeneous. A parametric test, namely; One Way ANOVA, was used to evaluate the differences among groups, and this gave a p-value of 0.000 ($p < 0.05$), which indicates a significant difference in the fibroblast cell cultures of each group. Additionally, subsequent tests involving LSD revealed that the results for the bovine amniotic membrane group were significantly different from the media control, yielding a p-value of 0.000 ($p < 0.05$). However, no significant difference was observed between the cell control and the BAM group, with a p-value of 0.868 ($p > 0.05$).

Effect of BAM on osteoblast cell viability
The results of the MTT assay for osteoblast cells after BAM intervention showed a viability of 91.73%, indicating that BAM was non-toxic. The microscopic observation of osteoblast cells after BAM intervention revealed blue formazan crystals in the osteoblast control cell group (Figure 1c) and the BAM group (Figure 1d). This indicates the number of viable osteoblast cells after the MTT assay conducted 24 hours post-intervention. The proliferation of osteoblast cell is influenced by several growth factors present in the bovine amniotic membrane, including insulin-like growth factor-1 (IGF-1) and transforming growth factor-β (TGF-β). Insulin-like growth factor-1 (IGF-1) is the most prevalent growth factor in the bone matrix,

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influencing growth, differentiation, metabolism, and various other physiological processes.[25,26]

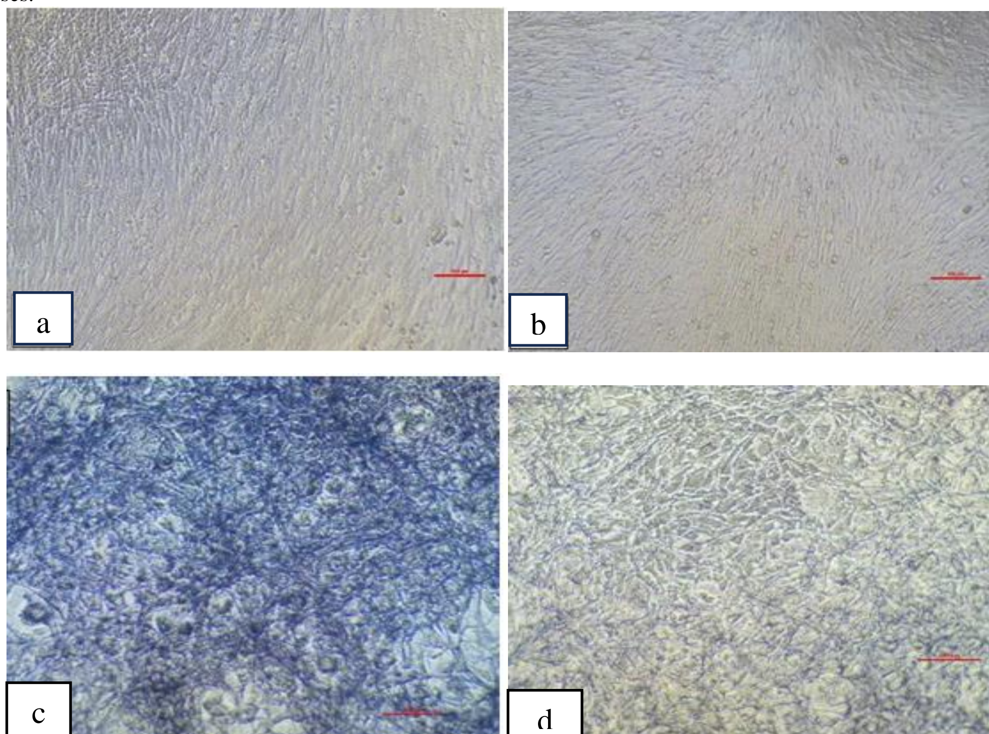

Figure 1: Fibroblast and osteoblast cells observed under a light microscope after the MTT assay. **(a)** Fibroblast cells appear purplish-blue in control cells group, **(b)** Fibroblast cells appear purplish-blue in BAM group, **(c)** Osteoblast cells appear blue in control cells group, **(d)** Osteoblast cells appear blue in BAM Group

In addition, Transforming Growth Factor-β (TGF-β) in the amniotic membrane has been proven to play a role in the Runx2 signaling pathway and initiate bone morphogenetic protein (BMP) synthesis signals in osteoprogenitor cells. This process promotes osteoblast differentiation and induces osteoclast apoptosis, thereby inhibiting bone resorption and enhancing osteoblast differentiation through the Runx2 signaling pathway.[27,28] these observations indicate that BAM is safe and suitable for use as a bone-regeneration material, particularly in the field of dentistry.[29,30]

After undergoing the Shapiro-Wilk normality test, the data was found to be normally distributed. To assess the homogeneity of the osteoblast cells, Levene's test was conducted. The results indicated that the data was homogeneous, with $p = 0.076$ ($p > 0.05$). Subsequently, a parametric test using One-way ANOVA was conducted to evaluate the differences between the groups. The results showed a significant difference in the osteoblast cell culture among the groups, with $p = 0.000$ ($p < 0.05$). Additional post hoc tests using LSD revealed that the bovine amniotic membrane group differed significantly from the media control group ($p = 0.000$; $p < 0.05$), but no significant difference was found between the BAM group and the cell control group ($p = 0.381$; $p > 0.05$).

The bovine amniotic membrane contains several growth factors, including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), transforming growth factor beta (TGF-β), and vascular endothelial growth factor (VEGF), all of which are essential for normal tissue and wound healing.[31,32] High viability and insignificant differences in the results indicated that BAM is safe and non-toxic to osteoblast and fibroblast cells.[18,33]

Conclusion

The findings from this study have shown that osteoblast and fibroblast cells exposed to the bovine amniotic membrane have a high level of cell viability, with percentage viability of 98.44% and 91.73% for fibroblast and osteoblast cells, respectively. Consequently, it can be said that BAM is not toxic to osteoblast and fibroblast cells. Future studies in animals and clinical trials are required to determine whether disparate outcomes could affect the effectiveness of the bovine amnion membrane.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them

Acknowledgments

The authors are grateful to the Dental Research Center, Faculty of Dental Medicine, Airlangga University, for providing sample authentication services.

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