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Short Communication

Analysis of morphology, cytotoxicity, and water content characteristics of freeze-dried amnion membrane from human and bovine

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Abstract

Placenta tissue has biological advantages, including anti-inflammatory, anti-bacterial, anti-fibrotic formation, and immunomodulatory properties. The amnion membrane (AM) is an inner side membrane of the placenta that faces the fetus. The main sources of amnion are humans and animals, with bovine being one of the significant sources. The aim of this study was to analyze the morphology, cytotoxicity, and water content characteristic of freeze-dried amnion membrane (FD-AM) from humans and bovines to measure the safety and compatibility of bovine FD-AM as an alternative to human FD-AM. This study is an observational cross-sectional study. Samples were divided into two groups: human FD-AM and bovine FD-AM groups. Both groups were examined for morphology characteristics by scanning electron microscopy (SEM), cytotoxicity by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) analysis, and water content by drying through moisture analyzer device. The morphology characteristics of bovine FD-AM and human FD-AM, as observed through SEM, showed similar results of a smooth, flat surface with no cavity and were well dehydrated. MTT assay analysis on both groups demonstrated cytocompatibility with cell viability exceeding 70% in the control group. However, human FD-AM showed a higher number of viable cells (0.19±0.01) compared to bovine FD-AM (0.12±0.03), with a statistically significant difference ($p<0.05$). The water content analysis revealed that both groups met the standard, with levels below 10%. While bovine FD-AM (7.19±0.45%) had slightly higher water content than human FD-AM (6.79±1.0%), the difference was not significant ($p>0.05$). Both human FD-AM and bovine FD-AM showed good results in morphology, cytotoxicity, and water content characteristics and compatibility. In conclusion, bovine FD-AM might be considered as an alternative to human FD-AM.

Keywords: Cytotoxicity, morphology, water content, human FD-AM, bovine FD-AM

Introduction

The human placenta is an easily accessible, affordable, and ethically acceptable source of raw material because it is a transient important organ typically disposed of as medical waste. The placenta possesses advantageous biological characteristics essential to the healing process, including angiogenic, anti-inflammatory, antibacterial, antifibrotic, and immunomodulatory with low immunogenicity [1]. Placental tissues exhibit distinct extracellular matrix (ECM)

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characteristics complementary to the desirable biological features. These properties include elasticity, stiffness, and tensile strength [2]. The amnion membrane (AM) is part of the placenta, which appears as a transparent thin layer of membrane on the inner side of the placenta that faces the fetus. AM envelops the fetus and covers the amniotic fluid-filled cavity [3]. Clinically, AM has been extensively used in various medical fields, particularly in treating burns and preventing tissue adhesion during surgery due to its suppression of pro-inflammatory cytokines, anti-inflammatory, anti-bacterial, anti-fibrotic, low antigenicity, and immunomodulatory properties [4].

Nowadays, human amnion is predominantly used in medical applications. However, the availability of willing donors limits the use of human amniotic transplantation, similar to other human tissue materials [5]. Amnion from animal sources may serve as an alternative. However, it has primarily been studied for veterinary purposes despite its potential benefits in certain situations. For instance, amniotic membranes derived from bovine [6], equine [7], porcine [8], and canine [9] sources have been studied. Therefore, the utilization of heterografts, such as bovine amnion, could offer a significant source of alternative material, similar to other collagen-based materials for surgical purposes.

The bovine amnion has the advantages such as a larger surface area and fewer legal or ethical concerns compared to human amnion [10,11]. Bovine amnion also has potential for application in regenerative medicine and reconstructive surgery [11]. However, the characteristics of bovine amnion must be studied for its safety and compatibility before clinical application. Previous studies of physical and biomechanical aspects of human amnion and bovine amnion found that bovine amnions have significantly greater tensile strength and thickness compared to human amnion [12]. The aim of this study was to analyze the characteristics of morphology, cytotoxicity, and water content of freeze-dried amnion membranes (FD-AM) derived from human and bovine to measure the safety and compatibility of bovine FD-AM as an alternative to human FD-AM.

Methods

Study design and setting

This study was an observational cross-sectional study with an in-vitro design that analyzed the characteristics between bovine FD-AM and human FD-AM. Both samples of human FD-AM and bovine FD-AM were processed and prepared by the Cell and Tissue Bank-Regenerative Medicine Center of Dr. Soetomo General Academic Hospital in Surabaya, East Java, Indonesia as reported in previous studies [13,14]. The FD-AM was then examined for morphology using scanning electron microscopy (SEM), cytotoxicity using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay, and water content measurement by drying it with a moisture analyzer device. Ethical clearance was approved by the Ethics Committee of Dr. Soetomo General Academic Hospital, Surabaya, East Java, Indonesia.

FD-AM production, preparation, and sterilization

Amnions were procured from fresh placentae of human donors (aged 18–42 years) and local domestic bovine at a local farm with a minimum age of 24 months and a breeding history of five times or less. All human donors provided written consent. After evaluation, it was determined that the donors had no history of substance misuse, syphilis, hepatitis B, hepatitis C, or HIV. Moreover, this study excluded placentae from patients with pregnancy problems or those contaminated with meconium.

Placentas were aseptically removed from the delivery room during normal labor or the operating theater during cesarean birth. The placenta must then be examined for damage indicators, such as discoloration, presence of debris or other impurities, odor changes, and other issues. If the placenta was deemed healthy, the separation of the amnion membrane and chorion layer was performed. Subsequently, regular saline (0.9% NaCl) was used to eliminate mucus, dirt, and blood clots. The amnion membrane was then placed in a sterile container with 0.9% NaCl solution, sealed, labeled, and stored in a cool box at -4°C for delivery to the Dr. Soetomo General Academic Hospital's Cell and Tissue Bank.

The Tissue Bank assessed the fresh amnion membranes for sterility, container condition, and completeness of the administrative documentation. The amnion membranes were then stored at -20°C in a quarantine cabinet. In a separate clean room environment for humans and bovines, the amnion membranes were treated using sterile devices on the table with sterile cloth. After reaching room temperature, which took approximately ten minutes, the amnion membrane was washed with normal saline (0,9% NaCl) and transferred to the processing pan. The amnion membranes were then decontaminated by soaking in a 0.05% NaOCl solution for ten minutes, followed by ten changes of the 0.9% NaCl solution every fifteen minutes. The amnion membranes were then placed in a water bath shaker (Julabo SW23) with 0.9% NaCl at room temperature. The cleaned amnion membranes were spread out on a sterile piece of gauze with the chorion side facing the gauze, followed by a deep-freezing process for at least 24 hours at -80°C to create FD-AM. Subsequently, the amnion membranes underwent lyophilization (using Lyophizer Lyovoc GT2) at temperatures between -40°C and -50°C for six to eight hours or until the water content of the membranes reached 6–7%. After lyophilization, amnion membranes were sliced to the appropriate size and stacked in three layers of vacuum-sealed polyethylene plastic. This procedure was done in a cabinet with laminar airflow [13,14]. The entire process of amnion membrane preparation was conducted in the Cell and Tissue Bank-Regenerative Medicine Center of Dr. Soetomo General Academic Hospital in Surabaya.

After the packaging process, FD-AM underwent sterilization. Sterilization was performed by exposing the FD-AM under irradiation with 25 kGy gamma-ray (Co60) (Kimura, Chemical Plants Co., Ltd, Osaka, Japan) in BATAN (National Nuclear Energy Agency of Indonesia), Jakarta. Sterilization was performed on all samples to ensure no contamination [13].

Morphology characteristics using SEM examination

All samples were prepared and dried before SEM examination to obtain a clear image. Samples were washed with PBS and then fixated with 2.5% glutaraldehyde for two hours at 40°C to preserve and stabilize the original structure. Dehydration was performed with graded alcohol concentration (50%, 70%, 80%, 90% until 100%) to remove water, followed by coating with osmium to increase surface conductivity during SEM examination [15]. The morphology structure of the amnion was analyzed using SEM (HITACHI FlexSEM 1000, Hitachi High-Tech, Tokyo, Japan) on 1,000×, 2,500×, 5,000×, and 10,000× magnification in the SEM Laboratory of Mechanical Engineering Department, Institut Teknologi Sepuluh Nopember in Surabaya, Indonesia. The surface morphology was evaluated qualitatively from images taken in different magnifications.

Cytotoxicity examination with MTT assay

The cytotoxicity of the amnion membrane was examined using MTT assay as described by the International Organization for Standardization (ISO) 10993–5:2009 [16]. In brief, the amnion membrane was cut into 1×1 cm pieces and placed in a well, followed by seeding with 0.3×105 rabbit adipose-derived mesenchymal stem cells (AdMSC) on the amnion surface and incubated for 30 minutes. MEM-Alpha+FBS 10% growth medium was added and incubated for 24 hours. Following the 24-hour incubation period, each well was washed with 500 µL/well PBS before adding 500 µL/well MTT solution and then incubated for an additional four hours at 37°C.

After aspirating the medium and cleaning the well with PBS, the well was dried for two hours before adding 500 µL/well of dimethyl sulfoxide (DMSO). The microtiter plate was set up on a shaker to dissolve the dye. Following the dissolution of the formazan crystals, the optical density (OD) value was measured spectrophotometrically by enzyme-linked immunosorbent assay (ELISA) reader using an ELX800 UV universal microplate reader (Bio-Tek Instruments Inc., Vermont, USA). The reference wavelength was set at 570 nm for measuring the sample OD value, which indicates cytotoxicity and cell proliferation with rabbit AdMSC as cell control [14].

Water content measurement

Water content was measured by drying with Moisture Balance BEL Engineering Series i-ThermoG L-M device (BEL Engineering Sri, Monza, Italy) as a moisture analyzer. Each sample of amnion was cut into uniform sizes of 2.5cm × 5cm and then placed in a sample plate before

measurement with the device and underwent three replications. Each sample plate was cleaned, and the device was cooled down for 25 minutes before testing the next sample.

Data analysis

The morphology structure of human FD-AM and bovine FD-AM were described based on pictures taken from SEM examination. The cytotoxicity data were analyzed with MTT assay based on OD value measured from human FD-AM, bovine FD-AM, and cell control of Rabbit AdMSC. MTT assay data were then described based on the OD value percentage of human FD-AM and bovine FD-AM compared to cell control.

The water content data were calculated in percentage (%) of water content and then described as whether the examination result met the required standard for water content. The data from the MTT assay and water content results were then processed through an analytical study using IBM SPSS Statistics software (IBM Corporation, Armonk, NY, USA). The Shapiro-Wilk Normality test was initially performed and followed by an independent t-test for normally distributed data.

Results

Morphology characteristic results

Morphological evaluation through SEM examination of human FD-AM (**Figure 1**) at 1,000× magnification shows a layer on the surface, while the surface looks smooth and well dehydrated. There was no difference from 2,500×, 5,000×, and then up to 10,000× magnification, where multiple indentations were found on the surface without any visible cells and no cavity.

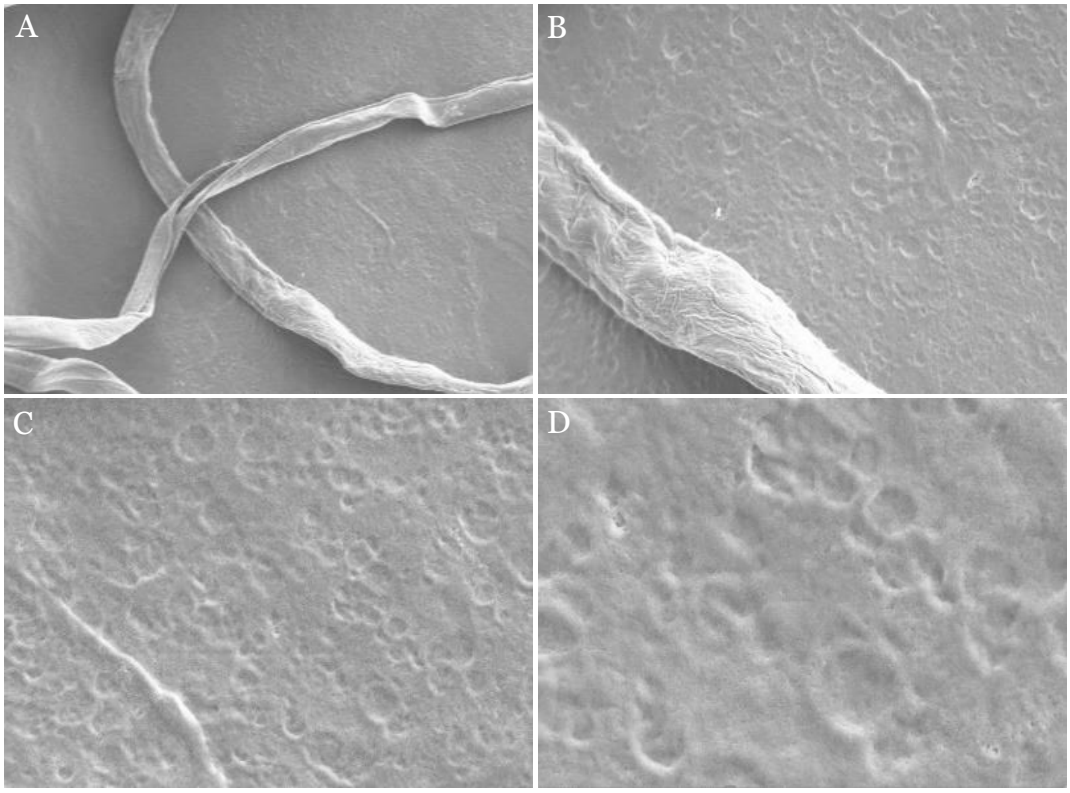

Figure 1. Images of scanning electron microscopy (SEM) examination for human freeze-dried amnion membrane (FD-AM) at different magnifications: (A) 1,000×; (B) 2,500×; (C) 5,000×; and (D) 10,000×.

SEM evaluation for bovine FD-AM (**Figure 2**) at 1,000× magnification showed multiple cell clumps scattered throughout the field of view with smooth and flat scaffold. There was no difference from 2,500×, 5,000×, and then up to 10,000× magnification, where the cell lumps were more visible from greater magnification. The surface of the amnion looked flat, smooth, and well dehydrated. No indentations or cavities were found on the surfaces.

MTT assay results

MTT Assay results are presented in **Table 1**. From these results, the number of cells detected in the human FD-AM group (mean OD=0.1868) was higher by almost 1.6 times compared to the bovine FD-AM (mean OD=0.1163). Based on the OD value calculation, human FD-AM (mean OD=0.1868) is higher than cell control (mean OD=0.1617), which signifies no cytotoxicity of human FD-AM with a living cell percentage exceeding 100%. However, the OD value of bovine FD-AM (mean OD=0.1163) is lower compared to cell control (mean OD=0.1617), with 72% of the living cell percentage.

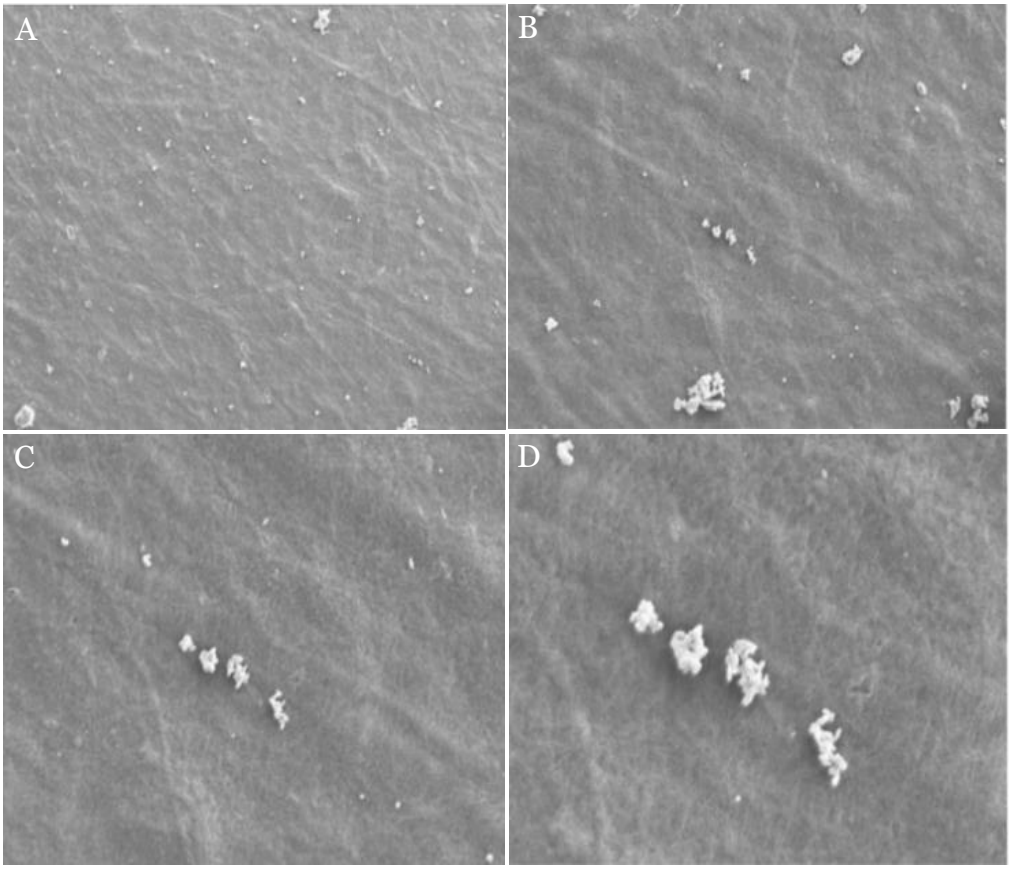

Figure 2. Images of examination results using scanning electron microscopy (SEM) for Bovine human freeze-dried amnion membrane (FD-AM) samples. The images appear at different magnifications: (A) 1,000×; (B) 2,500×; (C) 5,000×; and (D) 10,000×.

Based on **Table 1**, the normality test for bovine FD-AM group and human FD-AM group was analyzed using Shapiro-Wilk. The calculation result showed that the data was normally distributed (p>0.05) for both groups, with p=0.114 for bovine FD-AM and p=0.513 for human FD-AM. An Independent t-test was performed, which resulted in a significant difference between the results of the MTT Assay on the bovine FD-AM group and human FD-AM group with a p-value of 0.003 (p<0.05).

Table 1. MTT assay results

Group	Bovine FD-AM		Human FD-AM		Cell control	
	OD	%	OD	%	OD	%
Replication						
1	0.1030	58.6109	0.2059	117.1933	0.1757	100
2	0.0829	53.1942	0.1724	110.6581	0.1558	100
3	0.1038	65.1083	0.1821	114.2454	0.1594	100
4	0.1449	88.5662	0.1870	114.3076	0.1636	100
5	0.1473	95.4001	0.1870	121.1208	0.1544	100
6	0.1038	63.9445	0.1862	114.7304	0.1623	100
7	0.0829	52.2219	0.1765	111.2197	0.1587	100
Mean	0.1163	72.1800	0.1868	115.5100	0.1617	100

Group	Bovine FD-AM		Human FD-AM		Cell control	
	OD	%	OD	%	OD	%
Standard deviation	0.0284	18.7234	0.0122	3.9019	0.0086	0.00
p-value*	0.003					

FD-AM: freeze-dried amnion membrane; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; OD: optical density
[a] Analyzed using t-test
*Statistically significant at $p<0.05$

Water content

Water content analyses for both samples are presented in **Table 2**. On average, the bovine FD-AM group (7.192% ± 0.45%) was found to have a slightly higher water content than the human FD-AM (6.796% ± 1.0%). The normality test for bovine FD-AM group and human FD-AM group was analyzed using Shapiro-Wilk. The calculation results revealed that the data was normally distributed ($p>0.05$) for both groups, with $p=0.636$ for bovine FD-AM and $p=0.399$ for human FD-AM. An Independent t-test was performed, which resulted in no significant difference between the results of the water content in the bovine FD-AM group and human FD-AM group with *p*-value of 0.166 ($p>0.05$)

Table 2. Water content results

Samples	Replications (%)			Water content (%)	*p*-value*
	1	2	3		
Human FD-AM	7.929	6.438	6.022	6.796±1.0	0.166
Bovine FD-AM	6.702	7.291	7.585	7.192±0.45	

FD-AM: freeze-dried amnion membrane
[a] Analyzed using t-test
*Statistically significant at $p<0.05$

Discussion

Amnion membranes have been known to promote epithelization, reduce fibrotic tissue formation, angiogenesis, and inflammation. Amnion membranes, which contain an abundance of collagen type I, II, and III, also have growth factors and cytokines important for the healing process and have anti-bacterial properties with no immunogenicity that may cause allergic processes [1]. Those properties of amnion membranes resulted in the potential of amnion membranes application as a biological dressing for various types of wounds, including diabetic ulcers, tendon repair surgery, and prevent tendon adhesion [17,18]. Bovine amnion membranes have several advantages compared to human amnion membranes. Bovine amnion membranes have a larger surface area with a similar gestation time (280–290 days), and less ethical or law issues compared to human amnion, which needs informed consent from the subject, and some religions do not approve of amnion usage [19].

The safety and compatibility of human and bovine amnion membranes must be cleared before clinical application to humans. In this study, cytotoxicity is evaluated by measuring cell viability through MTT assay [3]. MTT assay result revealed both human and bovine amnion membranes have good results exceeding 70% cell viability, which is in line with the previous study [20], therefore it is cytocompatible [21]. However, human amnion membranes have significantly better results in cell viability compared to bovine amnion membranes.

SEM evaluation of human and bovine amnion membranes showed no difference in surface texture and morphology. Both human and bovine amnion membranes showed flat, smooth surfaces with dry and dehydrated surfaces, which aligned with the water content examination of both group samples. Morphology and surface texture of the membranes are important for cell proliferation due to affecting the interaction between integrin-ligand cells and the environment directly. Therefore, the mechanical properties of the membranes greatly influenced cell properties formation (morphology, interconnectivity, and tissue diameter) [22].

The water content of amnion is required to be below 10%, preferably between 4–7%, to accommodate long-term storage and prevent free radical formation during the sterilization process [3,23]. High water content may also promote the growth of contaminants such as fungi and bacteria [3]. In this study, both human and bovine amnion met the requirement value of

water content below 10%, with bovine amnion slightly higher than human amnion but no significant difference.

Moving forward, future applications of bovine amnion membranes include application in tissue engineering [2], reconstructive surgery [11], wound care [24], tendon [25] and nerve healing [26]. However, further research is necessary, which involves animal trials and then continues to clinical human trials. Future studies could also cover efficacy comparisons between bovine amnion membranes and human amnion membranes in animal trials before proceeding to clinical human trials. This comparison would help establish the viability of bovine amnion membranes as an alternative to human amnion membranes and determine whether the morphology, cytotoxicity, and water content characteristics identified in this study might influence efficacy during animal and clinical trials.

Conclusion

This study concludes that both human and bovine FD-AM showed favorable results in morphology, cytotoxicity, and water content characteristics and compatibility. However, a significant difference was noted in the cytotoxicity aspect of human FD-AM, which is better than bovine FD-AM. While the water content aspect of bovine FD-AM is slightly higher than that of human FD-AM, there was no significant difference. SEM examination of freeze-dried human and bovine amnion shows similar results in texture and morphology. Therefore, bovine FD-AM might be considered as an alternative to human FD-AM. However, future research in animal and clinical trials is necessary to find whether differences in cytotoxicity results might impact the efficacy between human FD-AM and bovine FD-AM.

Ethics approval

The ethical approval for this study was obtained from the Ethical Clearance Committee of the Faculty of Medicine, Airlangga University - Dr. Soetomo General Academic Hospital, Surabaya, Indonesia (Reference number: 0988/LOE/301.4.2/VIII/2022).

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Competing interests

The authors declare no conflict of interest regarding the submission of this paper.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

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