Histoanalysis and sterility assay of fresh human amniotic membrane for use in urogynecology

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Abstract. Human amniotic membrane has long been used as a biomaterial for wound/burn and varicose/diabetic ulcer dressing to prevent endometrial adhesion, as a graft for the cornea, periosteum, and bladder, or as a material to generate a neovagina. Several clinics in Indonesia opt for fresh amniotic membrane for grafts because it is widely available and easy to obtain. This study investigated the reliability of using fresh amniotic membrane as a biomaterial. Placenta samples were obtained from patients who underwent cesarean without complications, and human amniotic membrane was separated from the chorion and cut into a 3 × 3 cm² size. Specimens were categorized into two groups: before (0.9% NaCl) and after (2 g.L⁻¹ kanamycin) antibiotic wash as the control and fresh amniotic membrane groups, respectively. Cells in the amniotic membrane matrix were visualized using hematoxylin and eosin and DAPI staining. DNA in the matrix was quantified using Quick-DNA™ Miniprep Plus Kit, measured using a BioDrop nanospectrophotometer. DNA length was determined using gel electrophoresis. Sterility assay was performed using nutrient broth, followed by plating on agarose to identify any strain. Sections of both groups showed an intact cuboidal epithelial monolayer at the fetal surface. DNA residue in the matrix after NaCl and kanamycin washes was 41.36 ± 8.48 ng.mg⁻¹ and 42.53 ± 9.57 ng.mg⁻¹ tissue weight, respectively. All samples were sterile. Fresh amniotic membrane for clinical use might not be ideal because nuclei were identified across the membrane and the dsDNA residue length was >200bp.

1. Introduction

The gold standard for transplantation is still the use of autologous tissues or organs. However, harvesting an autograft causes secondary surgical injuries and functional loss of the harvested site. An allograft is an alternative; however, HLA mismatch elucidates acute/chronic or hyperacute rejection and hinders its application. Patients transplanted with an allograft also cope with long-term immune-suppressant intake [1,2], and there is a risk of disease transmission [3,4]. Therefore, an autograft or allograft is not ideal.

Human amniotic membrane (HAM) has been used for centuries as a patch/matrix for wound/burn dressing [5]. HAM is found at the inner layer of the fetal membranous sac wall that surrounds the fetus during gestation and consists of a cuboidal epithelial monolayer that develops from the ectodermal part of the embryonic layer and a basement membrane that develops from the mesenchyme [6]. The
advantages of the amniotic membrane over other patches include its anti-bacterial property, capacity for anti-inflammation/pain relief [7] and low immune rejection effects [8]. The amniotic membrane is used as a temporary dressing for burns or skin loss [5-9] and diabetic ulcer [10] as a substitute cornea in keratitis ulcer [11] and as a material in mandibular-labial vestibuloplasty in oral-maxillofacial surgery [12].

Due to matrix shortage, clinicians particularly use fresh amniotic membrane, which is prepared by antibiotic washes. Amniotic membranes are also cost-effective and easy to access. Nonetheless, disease transmission is of concern for this practice due to a lack of adequate sterilization and immune rejection because allogeneic cells remain in the matrix, eventually leading to transplantation failure. Fresh amniotic membranes are used in vaginoplasty in congenital vaginal aplasia [13] and to prevent intra-uterine adhesion due to endometritis [14,15]. However, reports and follow-up data for its applications are limited. Despite various applications and impressive possession of amniotic membrane as a graft, the amniotic membrane is not widely used because its post-transplantation results are inconsistent. In the present study, we investigated the reliability of using fresh amniotic membrane for clinical use.

2. Methods

2.1. Sample procurement

Upon ethical approval (no 789/UN2F1/ETIK/2017), donors for HAM were selected and appropriate information was provided; the consents were then signed. HAMs (n = 6) were acquired from the placenta obtained from cesarean delivery, without pregnancy complication, and were washed in normal saline [0.9% NaCl (v/v)] for 15 min, followed by kanamycin wash (2 g.L⁻¹) for 15 min. The samples washed in NaCl only were used as controls. The specimens were cut into a 3 × 3 cm² size and stored at −20 °C until all the samples were collected.

2.2. Tissue fixation

Specimens for histology were cut into 5 × 5 mm² size, placed in a histology cassette, and soaked in 10% neutral-buffered formaline (v/v) for 3 h at room temperature, followed by a series of dehydrations and wax infusions. Each sample was positioned transversally into a histology mold, covered with molten wax, and left to solidify.

2.3. Histology staining

The wax blocks were sectioned at 5-µm thickness for all staining techniques, and every tenth section was analyzed. Upon staining, sections were initially dewaxed and rehydrated following the protocol described elsewhere. For hematoxylin and eosin (H&E) staining, slides were immersed in hematoxylin (Thermo Fisher Scientific) for 1 min and eosin (VWR International) for 3 min and then rinsed in running tap water. After a series of gradients of ethanol dehydration, the stained slides were covered with a DPX mountant (Thermo Fisher Scientific Ltd) and a cover slip, imaged using a brightfield upright microscope (Zeiss Axio Imager 2), and captured using a Zeiss AxioCam, through ZenBlue software. Sections stained for DAPI were immersed into a DAPI working solution (10 mg.mL⁻¹ DAPI in Trizma buffer) for 10 min, followed by three washes in phosphate buffer solutions (10 min), and mounted in 1,4-diazobicyclo-(2, 2, 2)-octane (Sigma-Aldrich) fluorescence mounting medium. Sections were imaged using a fluorescence microscope (Zeiss Axio Imager) through a DAPI filter.

2.4. DNA quantification

Each sample was weighed up to 25 mg, minced thoroughly using a sterile blade, and lysed in Proteinase K¹⁶ digestion at 55°C for 10 h. The DNA was isolated using Quick-DNA™ Miniprep Plus Kit (Zymo Research), and the total amount of the isolated DNA was quantified using a BioDrop DUO nanospectrophotometer (BioDrop). The length of the isolated DNA was determined using gel electrophoresis [1.5% (w/v)] stained with Syber®Safe DNA dye (Invitrogen).
2.5. Sterility assay
Specimens of each group (n = 6) were immersed in nutrient broth and incubated at 37°C for 14 days. The incubated media were plated on nutrient agar and fetal bovine serum agar, placed at 37°C for 14 days, and on Sabouraud agar at room temperature for 21 days.

2.6. Data analysis
Matrix cellularity was determined using H&E dye to visualize the presence of nuclei and to show the matrix histoarchitechture (matrix layer). Double-strained DNA in the nuclei was identified by DAPI and stained in bright blue. Sterility assay was analyzed from the growth in the nutrient broth and the microbe strain found in each agar type. DNA concentration from each extraction was quantified using a BioDrop DUO UV spectrophotometer, and the quantity was normalized for total elution volume (50 μL). Data are presented as the mean of total DNA per tissue weight (ng.mg⁻¹) ± 95% CI using the following equation:

\[
\text{DNA weight (ng) per tissue weight (mg) = mean absorbance} \times \frac{50 \, \mu\text{L}}{\text{mg tissue weight}}
\]

The length of the isolated DNA was determined by running the DNA in 1.5% agarose gel and imaging using an infinity gel documentation.

3. Results
H&E staining of HAM after kanamycin wash (Fig. 1B) showed a cuboidal epithelial monolayer on a dense eosinophilic basement, overlain on a loosely basophilic stroma. The specimen showed a lattice of nuclei, intact as an epithelial monolayer at the fetal surface, which was not different from the fresh HAM (Fig. 1A).

![Figure 1. Sections of human amniotic membrane stained using H&E: post-kanamycin wash (B) compared with the control (A); scale bars indicate 200x magnification. Images were obtained from a brightfield microscope. DAPI staining of the kanamycin wash (D) compared with the control (C). Scale bars indicate 200x magnification, and images were obtained from a fluorescence microscope. Red and white arrows point to the cuboidal epithelial monolayer facing the fetal site.](image-url)
Double-strain DNA in the nuclei of the epithelial cells was confirmed using DAPI staining, and they were found at the surface in both groups (Figs. 2A and B).

**Figure 2. DNA quantification of HAM after NaCl and kanamycin washes.** (A) DNA was quantified using a BioDrop nanospectrophotometer at 260-nm wavelength. Data were analyzed using Student’s t-test (ns = not significant, p > 0.05). Bars denote the mean (n = 6) ± 95% CI. (B) Gel electrophoresis of the fresh HAM and after kanamycin wash; a 100-bp DNA ladder (Geneaid) was used as the indicator. DNA bands are found at 3000 bp.

DNA quantification of HAM after kanamycin wash was 42.53 ± 9.57 ng.mg$^{-1}$ tissue weight, which was not different from the control (fresh HAM; 41.36 ± 8.48 ng.mg$^{-1}$ tissue weight). Data are presented as mean total DNA per mg wet tissue weight (n = 6) ± 95% CI.

Agarose gel showed that each group had a band found at 3000 bp. Sterility assay indicated no growth of any microbes.

4. **Discussion**

In the present study, HAM washed in normal saline (0.9% NaCl) followed by kanamycin wash did not cause matrix alternation, leaving the epithelial layer undisturbed. The remaining cellular materials in the graft matrix lead to graft rejection. Ideally, the biomaterial for graft transplantation should be acellular. According to Crapo (2011), a biological graft should have no nuclei visualized using H&E and DAPI stainings, and if DNA is present in the matrix, it should contain <50 ng.mg$^{-1}$ of total dsDNA per dry weight tissue and should have a length <200 bp [17]. HAM with an intact epithelial layer when transplanted might obstruct host’s cellular invasion and hinder matrix integration, whereas the DNA remains elucidate immune reaction, forms a calcification niche, and leads to matrix failure [18]. In the present study, the fresh HAM prepared for urogynecology patients still showed an intact epithelial layer. The DNA quantity in the fresh HAM with and without kanamycin washes was not different, with approximately 50-ng DNA per mg tissue in both groups. This quantification had isolated DNA from the HAM only because the sterilization assay of the HAM revealed no signs of contamination.

However, a limitation of the present study is that DNA quantification was isolated from a wet tissue. The tissue for DNA quantification should ideally be dried to remove all fluid content [19]. Perhaps, the DNA quantity in the present study was higher than what had been measured. Moreover,
the length of DNA remaining in the matrix exceeded the acceptable length of DNA that can be approved for a transplanted matrix. Neither normal saline nor antibiotic washes had 3000-bp length of DNA residue. The maximum permitted length of DNA in an acellular graft is 200 bp. The DNA donor elicits an immunological reaction in a recipient with unmatched HLA [20].

Reports have shown that fresh HAM reduces endometrial adhesion, better than dried HAM, although the results were still lower than satisfactory. Fresh HAM resulted in less recurrence in adhesion and higher recovery in menstrual flow after its application for 3 months compared with no graft [21]. Fresh HAM has also been reported to improve adhesion grade, menstruation, and uterine length compared with dried HAM. Fresh HAM use was accountable for 23.3% of cases showing spontaneous pregnancy after 4 months in most cases; twice was found among those with HAM application compared with those without application [15]. Fresh HAM use also showed lower pain scores in patients compared with an application of collagen matrix and was favorable over the collagen matrix in the average time for granulation in tissue formation (p = 0.036) and the resultant in scarring, although infections were found in both matrices [22].

Decellularized HAM using SDS has been shown to improve wound healing in full-thickness skin loss in mice. At an early stage, endothelial growth factor and alpha-smooth muscle actin were induced, but transforming growth factor beta-1 was reduced. This alleviated wound inflammation, promoted tissue regeneration, and relieved scar formation after 8 months [23]. Decellularized HAM using 5% ammonium chloride has been reported to protect burn patients from *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* infections [24].

Cells in the graft matrix inhibit host cell infiltration when transplanted. HAM after cellular removal (decellularized HAM) using 0.01% and 0.1% (w/v) SDS showed keratinocyte invasion and better angiogenesis, and it was able to absorb exudate when used as a graft to close a pharyngocutaneous fistula, leading to the acceleration of wound healing within 14 days [25]. Decellularized HAM has also been reported to improve limbal epithelial cell infiltration [26]. Others used acellular HAM (Acelagraft) and reported rapid epithelialization for chronic ocular surface disease compared with HAM with cells. Rapid epithelialization of the cornea was reported to reach up to 80% in the same day after application and complete closing of the ulcer within 24 days, which may be due to basement membrane exposure [27].

Therefore, a study to generate acellular HAM is needed. The technique is known as decellularization technology, deploying chemical, enzymatic, physical, and mechanical methods to devoid cellular materials without damaging the matrix histoarchitecture and its extracellular components.

5. Conclusion
The application of HAM in clinical use has been widely studied and has shown better responses than the application of other grafts. Acellular HAM has been reported to show rapid regeneration. However, those results are still below the satisfactory level. Perhaps, the existing cuboidal epithelial monolayer hinders host cellular integration and delays the regeneration process. Therefore, further study is needed to elucidate the method of removing cells without damaging the property and capacity of the membrane.

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Conflict of interest statement
The authors state that there are no conflicts of interest regarding the publication of this article.
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