A NOVEL USE OF AMNIOTIC BILAYER DRESSING CO-CULTURED WITH KERATINOCYTES AND STEM CELL AS SKIN SUBSTITUTE IN ADULT BURN PATIENTS

NOUVELLE UTILISATION DU PANSEMENT DE MEMBRANE AMNIOTIQUE BICOUCHE, EN COCULTURE AVEC KÉRATINOCYTES ET CELLULES SOUCHES, COMME SUBSTITUT CUTANÉ CHEZ LES ADULTES BRÛLÉS

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SUMMARY. Survival outcome of burn patients is increasingly higher with early excision and grafting. The problem after excisional debridement is the need for a skin graft or a temporary dressing. By using an amniotic membrane, which is a biological waste, we can limit the use of auto-skin grafts. A preliminary prospective study was conducted on adult patients with no other comorbidities admitted to dr. Cipto Mangunkusumo Hospital Jakarta Burn Unit between 2021–2022. Patients were divided into three groups, treated with early excision and skin grafting, the amnion bilayer dressing or amniotic bilayer dressings co-cultured with keratinocytes and human amniotic epithelial stem cells (hAESC). The patients were then followed up for two weeks and the resulting epithelization in each group was analyzed, including a skin biopsy for confirmation of the epidermal layer thickness. A total of six patients, two in each group, were included in the study. The amniotic bilayer dressings co-cultured with keratinocytes and stem cell group has a rapid rate of epithelization (10% / day) compared to the early excision and skin grafting and amnion bilayer group (7.14% / day). It also has the thickest epidermal layer (95.7μm - range: 67.4-124.02 μm) and the highest histological score (7.00) at two weeks follow-up. This preliminary study concluded that amniotic bilayer dressings co-cultured with keratinocytes and stem cells had an overall better outcome regarding epithelization rate, suggesting that further study is necessary to evaluate amniotic bilayer dressings co-cultured with keratinocytes and stem cell use in burn patients.

Keywords: amniotic membrane, burns, skin substitutes, stem cells

RÉSUMÉ. *L'excision-greffe précoce améliore considérablement la survie des patients brûlés. Le problème si situant après l'excision : la couverture, par autogreffe ou substitut cutané. La membrane amniotique, plutôt qu'être jetée, peut remplacer l'autogreffe. Nous rapportons une étude préliminaire conduite auprès de 6 adultes hospitalisés dans le CTB de l'hôpital Dr Cipto Mangunkusumo de Djakarta en 2021 et 2022, répartis en 3 groupes. Après excision, ils ont été recouverts par autogreffe, membrane amniotique bicouche (MAB) ou MAB avec coculture de kératinocytes et de cellules souches (MABCC). Ils ont été suivis pendant 2 semaines avant analyse histologique de l'épithélialisation permettant de mesurer l'épaisseur épidermique. Le rythme d'épithélialisation était de 10%/j dans le groupe MABCC contre 7,14% dans les autres groupes. Ce groupe avait aussi une couche épidermique plus épaisse (95,7μm- entre 67,4 et 124,02) et le score histologique le plus élevé à 2 semaines. Des études complémentaires sont nécessaires, dans le but de confirmer ces résultats.*

Mots-clés : *brûlure, membrane amniotique, substituts cutanés, cellules souches*

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Introduction

Burn injuries are one of the most common injuries in Indonesia. The mortality rate is also high, with the leading causes being sepsis and multiple organ failure.[1] The problems in treating burn injuries in Indonesia are due to the high mortality and morbidity rate. Fluid resuscitation is life-saving treatment for burn injury patients. After the patient is stabilized, the burn wound needs to be treated. Partial thickness burns and deeper burns will need surgical intervention. Surgical excision or debridement of eschar is needed to prevent infection and to have a vital tissue bed so the burn wound can be closed with skin grafts. The excision can be done tangentially or fascial. When the tissue bed is viable, the burn wound can be closed with grafts. This approach was published by Burke et al.[2] in the 1970s for burn patients with more than 30% TBSA, resulting in decreased length of stay and increased survival of the patients.[2-5] Currently, early excision is the gold standard treatment to remove biological nidus to prevent infection and burn sepsis. Most research resulted in a lower infection rate, and a decrease in length of stay and mortality rate in patients.[6] Autografts or skin grafts from the patient themselves will lower the infection rate, reduce pain, and promote early mobilization for the patient.

Defect closure after excision with grafts is incorporated in the early excision treatment. Grafts used can be autograft, allograft, xenograft and skin substitutes. Gold standards used are the split-thickness skin graft (STSG) originating from the patient themselves.[1] Using STSG has limitations considering it can only be meshed to approximately 4 times the original donor size. The Meek micrografting technique appears to be a potential solution. This technique was published in 1958 by C.P. Meek and it allows skin graft to expand, by cutting it into micrografts and expanding the skin graft into a ratio of 1:3, 1:4, 1:6 or 1:9.[7] However, the limitation of this technique is that it requires more advanced devices such as microdermatome, which is quite costly and not yet available in our burn unit. In cases with severe burns, allograft and xenograft are often used.[8] Another emerging modality is the usage of skin substitutes for burn patients. In this paper, the author tried to use a biological graft that is often considered biological waste, which is an amniotic membrane co-cultured with keratinocyte cells and human amniotic epithelial stem cells (hAESC).

Material and methods

A preliminary prospective comparative study was conducted on adult burn patients with partial thickness burn or deeper burn injuries admitted to dr. Cipto Mangunkusumo Hospital Jakarta Burn Unit between September 2021-March 2022. Patients who consented were divided into three groups, treated with early excision and skin grafting, the amnion bilayer dressing or amniotic bilayer dressings co-cultured with keratinocytes and stem cells.

Amnion bilayer scaffold preparation and bio-burden assay

The human amnions used in this study were obtained from the placenta of an elective cesarean, taken from donors without reaction/negative towards human immunodeficiency virus (HIV), cytomegalovirus and hepatitis B and C. The membrane was subsequently washed in PBS, followed by consecutive washes in Triton-X (0.1%; w/v) and sodium dodecyl sulphate/ SDS (0.05%; w/v), overlaid with fibrin, and then incubated at 37°C for 24 h. The scaffold was placed in a sterile pot at 4-8^{0}C.

All amnion bilayer scaffolds were analyzed for (i) bio-burden assay, using tryptone soy broth (Sigma, USA), Sabouraud dextrose agar (Sigma, USA), and blood agar (Merck, Germany), (ii) acellularity using H&E (Scytek, USA) and DAPI (Abcam, USA), (iii) biocompatibility assay using hAESC cells. All the scaffolds were found to be acellular, had no growth, and were biocompatible.

Cell preparation

Donors had a negative reaction to HIV, cytomegalovirus and Hepatitis B and C, and had been informed about the study.

The human amniotic epithelial stem cells/ hAESC were isolated from an elected cesarean amnion membrane, according to the published paper with modifications.[9,10] Briefly, the amnion mem-

brane was blunt separated from the chorion, washed subsequently in sterile Dulbecco Phosphate Buffer Serum/ DPBS-1x (Gibco, USA), followed by 50 ml of Versene solution (Gibco, USA) to remove blood residue. The amnion membrane was then spread fetal upright on a plate and incubated with TrypleSelect (Gibco, USA) 50 ml for 1 hour, 37^0C, and the maternal site was incubated using 2 $mg.mL^{-1}$ Collagenase-1 (Gibco, USA) and Hyaluronidase 66$IU.mL^{-1}$ (Thermo Fisher, USA) in DMEM 4.5 $g.L^{-1}$ (Sigma-Aldrich) for 1 hour, 37^0C. The membrane was scraped gently, neutralized with Trypsin inhibitor solution 1x volume (Gibco, USA), and DMEM supplemented with serum 10% (v/v).

Human keratinocytes/ hKerati were procured from the unburned part of the patient's skin: 1x2cm. Briefly, the skin was incubated in Dispase (Gibco, USA) 6.5 $mg.mL^{-1}$ in DPBS 1x (Gibco, USA) at 4^0C overnight, and the epidermis was then separated from the dermis, weighted, then minced, incubated in TrypleSelect (Gibco, USA) for 1 hour, at 37^0C. The cell suspension was then neutralized using a complete culture medium 1x volume, and the tissue debris was separated using a 70µm cell strainer (Biologics, Pakistan); the cells were collected using 300g centrifugation for 10 min.

Co-culture of hAESC and h-Kerati on an amnion bilayer scaffold

Both cell types were seeded onto the amnion bilayer three days before transplantation, with a seeding density of $5x10^5$ $cells.cm^{-1}$. The ratio of hAESC and h-Kerati was 9:1. The mixture cells were resuspended in the Epilife complete medium (Gibco, USA) and incubated at 37^0C in 5% CO_2 on air for three days.

Methods

The early excision and skin grafting was used as a control group and was then compared to the other two experimental groups. The second group was the early excision and treated with amnion bilayer dressings while the last group was the early excision and the amnion bilayer dressings co-cultured with keratinocytes cells and amniotic epithelial stem cells.

All patients were assessed clinically upon arrival at the emergency care for the % body surface area burned and were treated with fluid resuscitation for the first 24 hours according to parkland formula. After the patients were stabilized, they underwent early excision surgery within 5 days after the burn injury and underwent the second operation to close the burn wound three to four days afterward. After the first surgery, keratinocytes from the patient were isolated, and the seeding of keratinocytes and epithelial stem cells onto the amnion membrane takes time, approximately 3 days. This also gives time for burn wound conversion, thus increasing the accuracy of the burn area excised before grafting. After the seeding process is complete, the amnion bilayer dressings or amnion bilayer dressings co-cultured with keratinocytes cells and amniotic epithelial stem cells were applied to the patients on the second surgery. The patients were then followed up for two weeks.

The first dressing changes were done three days after the insetting of the skin graft for group 1, the amnion bilayer for group 2, or the amnion bilayer dressings co-cultured with keratinocytes cells and stem cells for group 3. Then the dressing changes were done on day 5, 7, 10 and 14 after the insetting operation.

On each day the rate of epithelization was noted and on day 14 a biopsy was taken in the center of the insetting area. Patients were discharged once complete epithelization occurred. The time taken until 100% epithelization in each group was noted. The biopsy taken on day 14 was then compared using a histological scoring for burns[11] and also the thickness of the epidermal layer noted.

Results

There were 6 patients admitted in this study with partial thickness burns, less than 50% TBSA. They were divided into three equal comparable groups. Patients subjected to split-thickness skin grafts taken from the patients themselves were considered group I, patients treated with amnion bilayer dressings were considered group II, and those who underwent treatment with amnion bilayer co-cultured with patient keratinocytes cells and amniotic epithelial stem cells were considered group III. Patient demography

can be seen in *Table I*. In this study, the age of the patients ranges from 20 to 51 years. The TBSA also ranges from 11-41.5% with partial thickness burns or deeper burns. Two-thirds of the subjects were males. The etiology of burns in this study was mostly flame burns, with the rest constituting only 16.7%, due to scald.

Table I - Sociodemographic characteristics of subjects

Variable	N = 6
Group	
I	2 (33.3%)
II	2 (33.3%)
III	2 (33.3%)
Gender	
Male	4 (66.7%)
Female	2 (33.3%)
Age (years)	
Mean ± SD	35 ± 11.5
%TBSA	
Mean ± SD	27.7 ± 12.2
Observation site	
Dorsum manus dextra	4 (66.7%)
Femoralis medial dextra	1 (16.7%)
Antebrachii dextra	1 (16.7%)
Grade	
II – III	6 (100%)
Cause of burn	
Fire	5 (83.3%)
Scald	1 (16.7%)

Healing time

Healing time is considered the time taken for 100% epithelization of the wound to happen. The area % of epithelization was noted every time dressing changes were made. All groups were treated with similar dressing change frequency, which was on days 3,5,7,10 and 14. The results for wound healing time can be seen in *Table II*. The rate for complete epithelization is faster in group III, although statistically not significant. Complete epithelization was achieved on day 10 for group III. We can see the clinical changes in each group, reported in *Fig. 1*. We can also see in group II that the amnion bilayer was resorbed mostly by day 7, but the epithelization process started and completed by day 14 in this group. Whereas in group III the amnion bilayer cocultured with keratinocytes cells and amniotic epithelial stem cells were not all resorbed and by day 10 the wound was dry, showing complete epithelization underneath the amniotic layer.

Table II - Rate of epithelization between groups

Group	Rate of epithelization (%/day)	p-value	Comparison	p-value
STSG	7.14 (7.14-7.14)	0.082*	STSG-Amnion Bilayer	1.000^
Amnion Bilayer	7.14 (7.14-7.14)		STSG-Amnion Coculture	0.083^
Amnion Coculture	10 (10-10)		Amnion Bilayer-Coculture	0.083^

*Kruskal-Wallis, ^Mann-Whitney, STSG: split-thickness skin graft

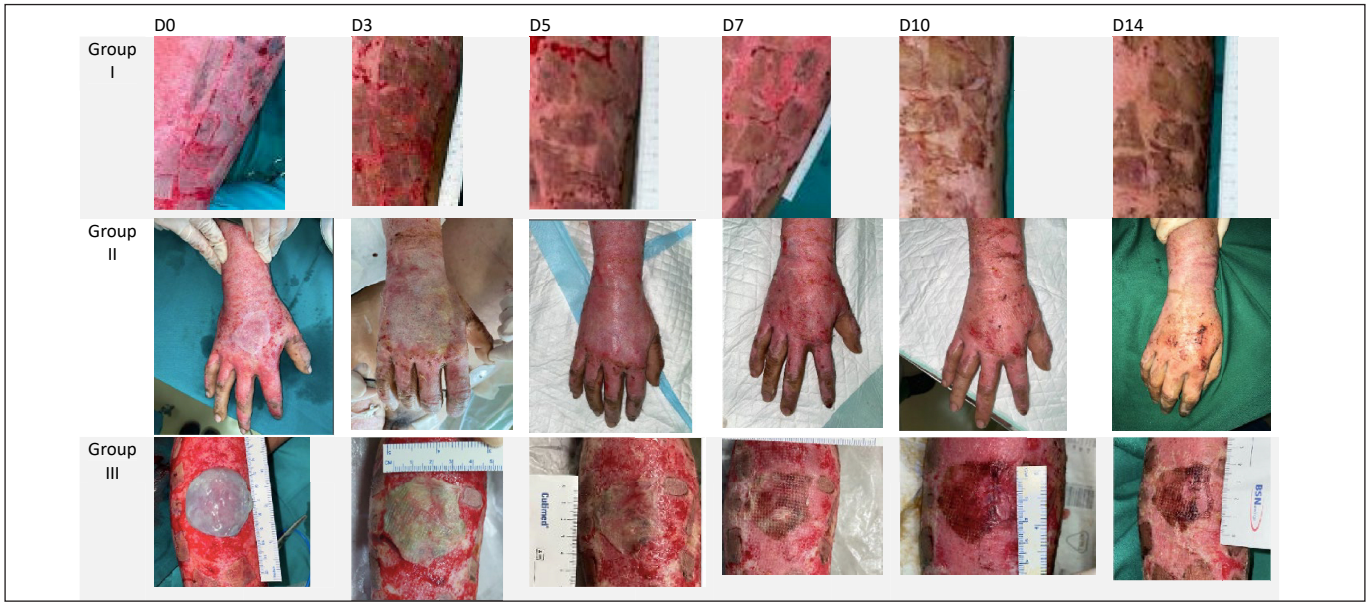

Fig. 1 - Clinical appearance of the wound at day 0, 3, 5, 7, 10 and 14 (n = 6). All wounds healed by the time biopsy was taken at day 14

Histological analysis of newly regenerated skin

To evaluate the new skin after the epithelization process, the researchers took a biopsy from the center of the insetting area with a size of 0,5x2 cm. To visualize the skin structures (epidermal and dermal layers) the tissue slides were stained with haematoxylin and eosin (H&E). A regenerated skin can be evaluated histologically using H&E staining. The normal bilayer skin structure has a relatively thin epidermis and highly cellularized dermis with hair follicles. A newly regenerated skin has a thickening of the epidermal layer and loose dermal matrix.[12] Complete reepithelization was observed in all groups on day 14 after insetting. The histological analysis also found epidermal and dermal layer which confirmed a newly regenerated skin. All groups showed intact but thickened epidermis layer, which can indicate a progression of healing. Epidermal thickness for each group can be seen in *Table III*. In comparison, we can also see each group has epidermal and dermal layers closely resembling normal skin in *Fig. 2*.

Histological scoring for burn wound healing[11] was also evaluated. The histological scoring evaluated the epidermal layer and divided it into 0-7 scores, with 7 representing complete epithelization with rete ridges and no crust attached. The dermis was also evaluated based on the proportion of adipose cells, inflammatory cells, fibroblasts, collagen depositions and formation of hair follicles with scores ranging from 0-7.[11] The histological scoring for each group can be seen in *Tables IV and V*. The epidermal layer scoring was highest in group III, with a score of 7. We can see the rete ridges were formed in this slide with keratin layers on top of the epidermal layer. The dermis layer histological scoring was highest in the STSG group.

Discussion

The aim of this study was to determine whether or not skin substitutes using amnion bilayer dress-

Table III - Epidermal thickness between groups

Group	Epidermal Thickness (µm)	p-value	Comparison	p-value
STSG	59.5 (49.6-69.4)	0.651*	STSG-Amnion Bilayer	0.439^
Amnion Bilayer	71.4 (69.3-73.5)		STSG-Amnion Coculture	0.439^
Amnion Coculture	95.7 (67.4-124.02)		Amnion Bilayer-Coculture	1.000^

*Kruskal-Wallis, ^Mann-Whitney, STSG: split-thickness skin graft

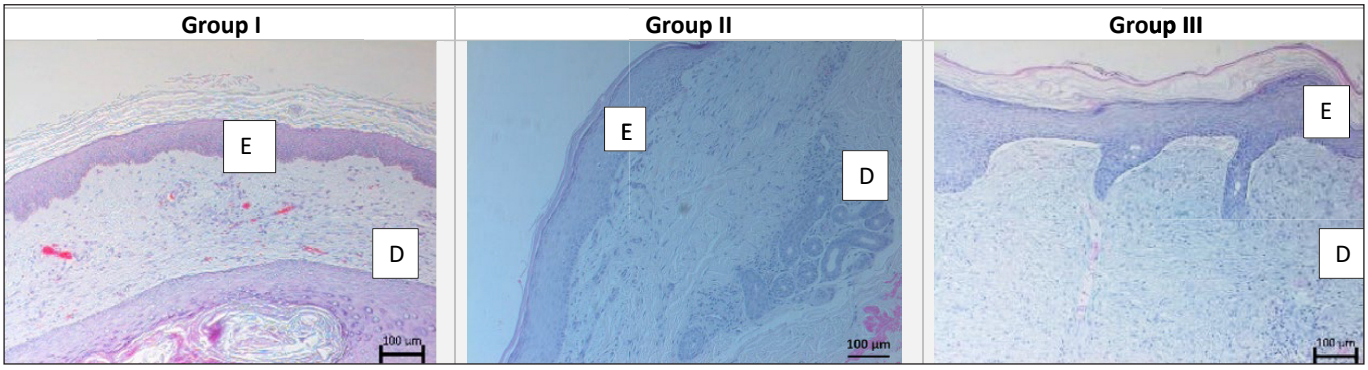

Fig. 2 - Histological cross-section of regenerated skin in each group (n = 6). H&E staining showed that the amniotic bilayer co-cultured with keratinocytes and stem cells showed more defined rete ridges on the epidermal layer compared to other groups. E: epidermis layer, D: dermis layer

Table IV - Epidermal histological scores between groups

Group	Epidermis Histological score	p-value	Comparison	p-value
STSG	6.50 (6-7)	0.535*	STSG-Amnion Bilayer	1.000^
Amnion Bilayer	6.50 (6-7)		STSG-Amnion Coculture	0.317^
Amnion Coculture	7.00 (7-7)		Amnion Bilayer-Coculture	0.317^

*Kruskal-Wallis, ^Mann-Whitney, STSG: split-thickness skin graft

Table V - Dermis histological scores between groups

Group	Dermis Histological score	p-value	Comparison	p-value
STSG	6.50 (6-7)	0.851*	STSG-Amnion Bilayer	0.683^
Amnion Bilayer	5.50 (4-7)		STSG-Amnion Coculture	0.683^
Amnion Coculture	6 (5-7)		Amnion Bilayer-Coculture	0.683^

ings can regenerate new skin after partial thickness burn injuries or deeper. Burn wound management is a daunting task for any surgeon when it is a case of major burns with little to no healthy skin donor site for skin grafting. There will be continuous loss of body heat, fluid and electrolytes due to the absence of skin barrier.[13] After an early excision surgery, the burn wounds need to be resurfaced. The standard practice is to use split-thickness skin graft from the patient or a biological dressing such as allografts or xenografts. However, using autograft has its limitations in terms of donor site availability in major burn patients. In general, an ideal skin substitute should have these properties: (1) inexpensive (2) long shelf life (3) readily available (4) not antigenic (5) good durability (6) flexible (7) prevent water loss (8) prevent bacterial colonization (9) can be conformed for irregular surfaces (10) can be fixated on the skin (11) grow with aging (12) applicable for one-step operation, and (13) no hypertrophic scar.[4] A novel skin substitute dressing using an amnion bilayer is proposed in this study. The biological dressing will create a barrier between the wound bed and the environment, thus preventing infections. Keeping the wound bed moist, the process of wound healing can be achieved. Amniotic membrane has good wound healing properties by promoting epithelization, reducing inflammation, neovascularization, and fibrosis. In partial thickness burns amniotic membrane has been proven to increase the re-epithelization process.[14]

The amniotic layer used in this study was classified into two groups, one is the amniotic bilayer which comprises the amnion and chorion layer. The other was the amniotic bilayer that has been co-cultured with patient keratinocyte cells and stem cells. The stem cells used were amniotic epithelial stem cells (AESCs) that originate from a full-term amnion membrane, commonly considered biological waste after a birth. AESC that has been isolated express several surface markers such as stage-specific embryonic antigen-3 and 4 (SSEA-3 and SSEA-4), octamer-binding transcription factor-4 (OCT-4), tumor rejection antigen 1-60 and 1-80 (TRA1-60 and TRA1-80). This shows that AESC has similar properties to embryonic stem cells (ESCs) and thus has good proliferation potency and self-renewal.[15] AESC also only expressed low human leukocyte antigens (including HLA-A, HLA-B, HLA-C, and HLA-DR), that have a role in rejection.[15]

The outcome of this study was as seen in *Fig. 1*, where complete re-epithelization was achieved. The rate for epithelization was higher in group III, which was 10% /day compared to group II with 7.14%/day. The biopsy taken by day 14 also showed a complete epidermal and dermal layer. The histological scoring of the epidermal layer was higher in group III. This can be seen because in group III the rete ridges were formed. These two parameters already showed the potential of amniotic bilayer dressing co-cultured with keratinocyte cells and stem cells.

Conclusion

This preliminary study concluded that amniotic bilayer dressings co-cultured with keratinocytes and stem cells had an overall better outcome regarding epithelization rate, suggesting that further study is necessary to evaluate amniotic bilayer dressings co-cultured with keratinocytes and stem cell use in burn patients.

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Ethical approval. The study was approved by the Faculty of Medicine Universitas Indonesia Ethical Committee for Medical Research (No. KET-866/UN2.F1/ETIK/PPM.002/2021) with research protocol number 21-06-0670 and performed in accordance with the principles of the Declaration of Helsinki.
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