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Short Communication

Can a combination of nanofat and freeze-dried human amniotic membrane enhance full-thickness wound healing? An animal study using rabbit models

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Abstract

Previous studies have explored nanofat stimulating tissue regeneration and maturation, promoting remodeling through its rich content of growth factors and stem cells; however, comprehensive data on its use in full-thickness wounds remains limited. The aim of this study was to evaluate the effectiveness of combining nanofat with freeze-dried human amniotic membrane (FDHAM) for treating full-thickness wounds in a rabbit model. An animal experimental study using a post-test control group design was conducted. Thirty-six male New Zealand white rabbits (*Oryctolagus cuniculus*) were randomly assigned to two groups: the experimental group (received a combination of nanofat and FDHAM) and the control group (received FDHAM alone). Each group was subdivided to evaluate effects on days 3 and 7. Macroscopic evaluations of wound healing, microscopic assessment of epithelialization and measurement of epidermal growth factor (EGF) levels in the wounds were conducted on days 3 and 7 post-injury. The present study indicated that the combination treatment significantly elevated EGF levels in the wounds on both days 3 and 7 (with $p<0.001$ for both assessment time points). The combination of nanofat-FDHAM did not significantly accelerate epithelialization on either day 3 or 7. This study highlights that combining nanofat with FDHAM did not significantly speed up epithelialization of full-thickness wounds within the first seven days; however, it notably increased EGF levels, suggesting that nanofat may enhance the wound's biological environment.

Keywords: Nanofat, stem cell, freeze dried amniotic membrane, full-thickness wounds, wound healing

Introduction

Wounds resulting from trauma, surgery, vascular or neuropathic conditions, pressure injuries, or malignancies disrupt the structure of tissues [1]. Full-thickness skin wounds pose infection risks and often result in permanent scars that are both functionally and aesthetically problematic [2]. The wound healing process consists of several phases: inflammation, proliferation, and maturation [2]. However, full-thickness wounds frequently become chronic, leading to poor healing and unsatisfactory scar formation [3].

Current clinical practices for managing chronic wounds include the use of amnion membranes for diabetic ulcers, burns, and postoperative wounds [4]. Amnion membranes accelerate healing by exhibiting antibacterial and angiogenic properties, enhancing epithelialization, stimulating granulation and neovascularization, reducing pain, facilitating scar-free healing, and minimizing immune rejection due to their strong adherence to wounds [5].

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Studies have found that amnion significantly reduces pain and accelerates epithelialization compared to tulle [6] and promotes faster epithelialization in superficial wounds [7]. Another study demonstrated that human amnion increases macrophage count and enhances wound healing more effectively than other dressings [8]. However, amnion is primarily effective for partial-thickness wounds and requires combination with other modalities, such as nanofat, for full-thickness wounds [8,9].

Nanofat stimulates tissue regeneration and maturation, promoting remodeling through its rich content of growth factors and stem cells [10-12]. A study demonstrated that nanofat injections increase dermal thickness and neovascularization in mice [13]. The combination of nanofat with amnion is expected to effectively reduce wound size by accelerating epithelialization, thereby decreasing patient morbidity, shortening treatment duration, and improving quality of life [14]. Previous studies have explored how nanofat stimulates tissue regeneration and maturation, promoting remodeling through its rich content of growth factors and stem cells [15-18]. However, comprehensive data on its use in full-thickness wounds remains limited. The aim of this study was to evaluate the effectiveness of combining nanofat with freeze-dried human amniotic membrane (FDHAM) for treating full-thickness wounds in a rabbit model.

Methods

Study design and setting

An animal experimental study using a post-test control group design was conducted at the Laboratory of the Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia, from December 2023 to January 2024. Male rabbits were randomly assigned into two groups: the experimental group, which received a combination of nanofat and FDHAM and the control group, which received FDHAM alone. Each group was further subdivided to assess the effects on day 3 and day 7. After full-thickness wounds were created, nanofat combined with FDHAM or FDHAM alone were applied. The outcomes—including the healing process (macroscopic assessment) and microscopic assessment, which included the quantification of epithelialization and epidermal growth factor (EGF)—were then evaluated.

Sample size calculation, allocation, and randomization methods

The sample size for the present study was calculated using the Federer formula, adjusted for a 10% mortality rate, resulting in the inclusion of 18 rabbits and a total of 36 full-thickness wounds. Each rabbit had two wounds, with one assigned to the experimental group (nanofat-FDHAM combination) and the other to the control group (FDHAM alone). The wounds were further subdivided into subgroups, with 9 wounds from each group evaluated on days 3 and 7 post-treatment. Randomization was achieved by assigning each rabbit a unique identifier and using a random number generator to allocate them to the experimental or control groups, as well as to assign the wounds to the designated evaluation time points. The present study was double-blinded to reduce bias; both the authors and veterinarians were blinded to group allocations, which were concealed in sealed envelopes. A third party, not involved in the present study, prepared the treatment solutions and applied them to the wounds according to the envelopes' instructions. Data analysis was conducted without knowledge of group assignments, ensuring blinding was maintained throughout the study.

Animal and eligibility criteria

Male New Zealand white rabbits (*Oryctolagus cuniculus*), aged 9–12 months and weighing 2500–3000 grams, were obtained from the Animal Laboratory Unit, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia. Exclusion criteria included female rabbits, those with prior experimental participation, and those presenting with skin abnormalities. Infection assessment was conducted through daily clinical observations for signs of infection, such as erythema, swelling, heat, pain, and discharge, supplemented by microbiological analysis of wound site swabs on days 3 and 7 post-treatment.

Dropout criteria included severe infections unresponsive to treatment, mortality during the study period, development of significant structural or functional abnormalities unrelated to

wound healing, or any condition severely compromising the animal's welfare. Each rabbit was monitored daily by a veterinarian for overall health and wound condition, with detailed records maintained and dropout rates tracked.

Animal acclimatization and animal husbandry

Rabbits were housed individually in cages measuring 50×50×60 cm under controlled environmental conditions to ensure optimal well-being. The ambient temperature was consistently maintained at 27°C, with natural light and a ventilation system providing a stable environment. Cages were cleaned daily by trained personnel, who removed soiled bedding, disinfected the enclosures, and replaced the bedding to prevent contamination. The lighting system was programmed to simulate a natural 12-hour light/dark cycle. The rabbits had constant access to a standard commercial diet consisting of pellets and hay, replenished daily to ensure freshness. Fresh water was continuously available through an automatic watering system, which was checked daily to ensure proper function and adequate hydration.

Daily health assessments were conducted by a veterinarian to ensure the well-being of the rabbits. Key health indicators included normal behavior, such as an active and alert demeanor, proper grooming, and a healthy appetite. Any signs of distress, including lethargy, changes in eating or drinking habits, abnormal feces, or indications of illness, were promptly addressed. Skin health was evaluated through visual inspection and palpation, with examinations for lesions, infections, or abnormalities such as erythema, swelling, or unusual texture. Rabbits presenting with any abnormalities during these assessments were excluded from the study to ensure the inclusion of only healthy animals, thereby maintaining the integrity of the experimental conditions.

Anesthesia and fat harvesting

Anesthesia was induced via intramuscular injection of ketamine (30 mg/kg) and xylazine (5 mg/kg). Following induction, prophylactic penicillin procaine (10,000 IU/kg) was administered intramuscularly to prevent infection. Adipose tissue was harvested by block excision from the abdominal region of each rabbit and processed into nanofat using the Tonnard technique and Tulip luer-lock system (Black Tie Medical, Inc., San Diego, California, USA). The fat was filtered through a nylon mesh to remove impurities and subsequently transferred into 1 mL syringes for injection.

Wound creation and treatment

Two full-thickness wounds, each measuring 2×2 cm, were created on the back of each rabbit using a #15 scalpel blade. The incisions were made with precision to ensure the complete removal of the skin and underlying tissue. The wound area was disinfected with a sterile solution to minimize the risk of infection. The wounds were then treated according to group assignments: one group received a nanofat-FDHAM combination, and the other was treated with FDHAM alone. The FDHAM was sourced from human donors, collected with informed consent, and processed in accordance with ethical guidelines. It was stored at -20°C until use to preserve viability and thawed immediately before application. Nanofat, prepared using the Tonnard technique, was injected directly into the wound bed using 1 mL syringes, with 0.2 mL administered per wound to ensure even distribution. FDHAM was then applied directly to the wound surface. Both treatments were covered with a transparent dressing to maintain a moist environment, promoting optimal healing conditions.

Incision closure and monitoring

After treatment, the wounds were covered with a transparent dressing to maintain a moist environment and reduce the risk of infection. The edges of the incisions were carefully approximated to promote optimal healing and minimize scar formation. The closure procedure employed sterile techniques and materials, including dressing application, to ensure the integrity of the healing process. The reliability of the nanofat was maintained through strict adherence to processing protocols and the immediate use of freshly prepared nanofat. Rabbits were closely monitored for signs of infection or abnormal healing. Health checks included daily assessments

of the wound sites for infection indicators and overall rabbit health. Any abnormalities led to immediate exclusion from the study to ensure that only healthy rabbits were included.

Biopsy specimen collection

On day 3 and day 7, biopsy specimens were harvested from both the central and peripheral areas of the wound to ensure representative sampling. Incisions were made using a #15 scalpel blade, and specimens were collected with sterile instruments. Each specimen was placed in a sterile container filled with formalin and transported to the laboratory for processing and analysis.

Outcome measurements

Macroscopic assessment

Wound healing was assessed macroscopically using the Visitrak digital system (Smith & Nephew Healthcare Ltd., Hull, UK), employing digital imaging technology to capture high-resolution images of the wound area. The images were analyzed to measure the rate of epithelialization by comparing wound sizes over time. The Visitrak system calculates the percentage of wound closure by analyzing the differences between the initial wound size before treatment and the evaluation day after treatment. Assessments were performed by trained personnel who ensured consistent image capture and accurate measurements.

Microscopic assessment

Histological evaluation of wound specimens was conducted to assess epithelialization. Biopsy samples were collected from the wound sites on days 3 and 7 post-treatment. The specimens were fixed in formalin, embedded in paraffin, sectioned to a thickness of 5 µm, and stained with hematoxylin and eosin (H&E) for microscopic examination. The sections were examined under a light microscope (Olympus Corporation, Tokyo, Japan) at magnifications of 100× and 400×, performed by a board-certified pathologist.

Quantification of epithelialization

The rate and percentage of epithelialization were quantified by measuring the extent of new epithelial tissue formation. This was accomplished by analyzing the stained tissue sections and assessing the proportion of the wound area covered by new epithelial cells relative to the total wound area. The results were expressed as the percentage of the epithelialized area in comparison to the total wound area. Key indicators of epithelialization included the presence of epithelial cells at the wound edges and the formation of a continuous epithelial layer.

Epidermal growth factor (EGF) quantification

EGF levels were measured using an enzyme-linked immunosorbent assay (ELISA). Biopsy specimens were homogenized, and EGF concentrations were determined with a commercially available rabbit epidermal growth factor ELISA kit (Gama Biotek, Indonesia). This kit employs specific antibodies to bind EGF, followed by a colorimetric reaction that correlates with the concentration of EGF in the sample. ELISA results were read using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA), and EGF levels were quantified in ng/mL.

Statistical analysis

SPSS version 25.0 software (IBM SPSS, Chicago, Illinois, USA) was employed for data analysis, with $p<0.05$ considered statistically significant. Continuous data were presented as mean and standard deviation (for normally distributed data) and median (minimum-maximum) for non-normally distributed data; categorical data were presented as frequency and percentages. Shapiro-Wilk test was utilized to assess data normality. To assess the association between groups, an independent sample t-test was employed for normally distributed data, while Mann-Whitney test was used for non-normally distributed data.

Results

Wound healing

On day 3, both treatment groups exhibited a median wound healing area of 4.00 cm², with no statistically significant difference (p=1.000) (**Figure 1** and **Figure 2**). By day 7, nanofat-FDHAM combination group showed a reduction in median wound healing area to 2.89 cm², while FDHAM alone group maintained a surface area of 4.00 cm² (p<0.05) (**Table 1**). These results indicate that the combination of FDHAM with nanofat significantly enhanced wound closure, beginning on day 7, compared to FDHAM alone.

Table 1. Comparison of wound healing area, epithelialization rate, epithelialization percentage, and epidermal growth factor levels between the experimental group (nanofat-FDHAM combination) and the control group (FDHAM alone) on days 3 and 7

Variables	Nanofat-FDHAM combination	FDHAM alone	p-value
Wound healing area (cm²), median (min-max)			
Day 3	4.00 (4.00–4.00)	4.00 (4.00–4.00)	0.100[a]
Day 7	4.00 (2.89–4.00)	4.00 (4.00–4.00)	0.060[a]
Epithelization rate (cm²), median (min-max)			
Day 3	0 (0–0)	0 (0–0)	0.100[a]
Day 7	0 (0–3)	0 (0–0)	1.000[a]
Epithelization percentage (%), median (min-max)			
Day 3	0 (0–0)	0 (0–0)	1.000[a]
Day 7	0 (0–0.1)	0 (0–0)	0.060[a]
Epidermal growth factor (pg/mL)			
Day 3, mean±SD	255845.33±66797.11	72077.44±6780.36	<0.001[b]
Day 7, median (min-max)	70000 (62174–82174)	108696 (92609–189130)	<0.001[a]

FDHAM: freeze-dried human amniotic membrane
[a] Analyzed using Mann-Whitney test
[b] Analyzed using independent sample Student t-test

Epithelialization rate

Macroscopic and microscopic evaluations indicated no significant differences in epithelialization between the nanofat-FDHAM combination (experimental) and the FDHAM alone group (control) on day 3 (**Figure 1**). Both groups exhibited similar levels of epithelialization, with the nanofat-FDHAM combination group showing a median epithelialization score of 0 (range: 0–3) and the FDHAM alone group having a median of 0 (**Table 1**). This finding suggests that no noticeable epithelial growth was present in either group at this early stage.

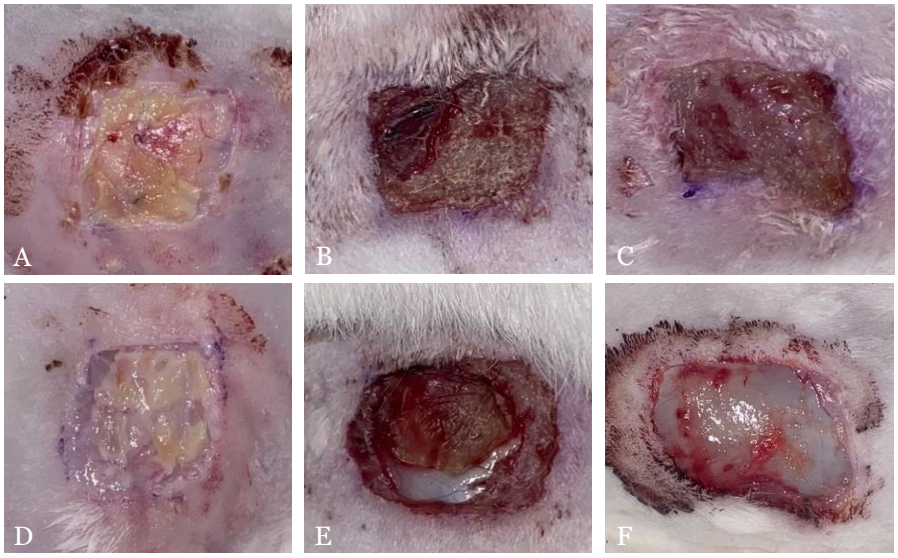

Figure 1. Representative pictures of wound healing area of full-thickness wound epithelialization on day 3: nanofat-FDHAM combination group (A, B, C) and FDHAM alone group (D, E, F).

By day 7, however, a significant difference emerged. The nanofat-FDHAM combination group exhibited visible signs of wound healing, including a marked reduction in wound size and improved epithelial coverage (**Figure 2**). This was further confirmed by the presence of a continuous epithelial layer forming from the wound edge, as observed microscopically (**Figure 3**). The median epithelialization for the nanofat-FDHAM combination group increased to 1 (range: 0–3), indicating partial epithelial growth and healing. In contrast, the control group maintained a median epithelialization of 0, demonstrating no visible epithelialization progress (**Table 1**).

These findings suggest that while both groups started with comparable conditions, the nanofat-FDHAM combination group exhibited enhanced epithelialization by day 7, attributable to the combined treatment of FDHAM with nanofat. Conversely, FDHAM alone group, did not demonstrate significant improvements in epithelialization or wound size reduction.

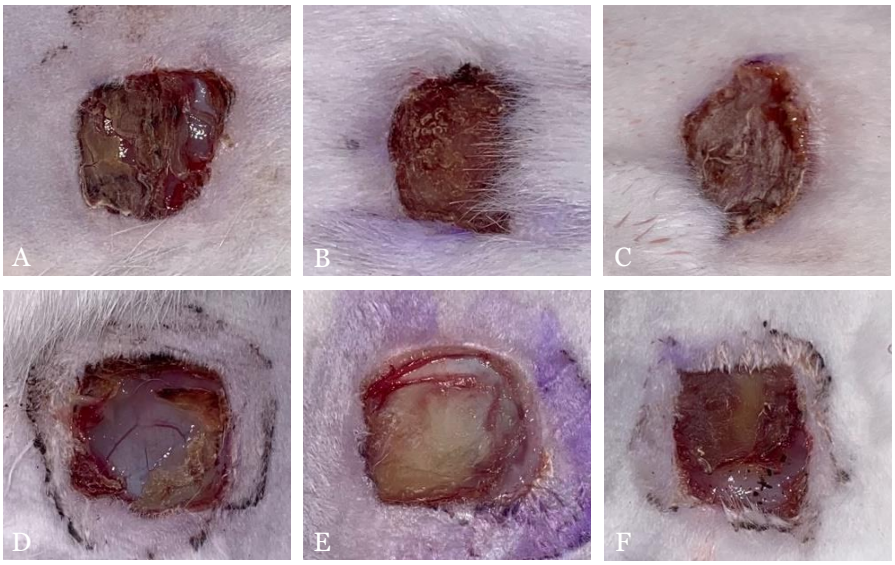

Figure 2. Representative pictures of wound healing area of full-thickness wound epithelialization on day 7: nanofat-FDHAM combination group (A, B, C) and FDHAM alone group (D, E, F).

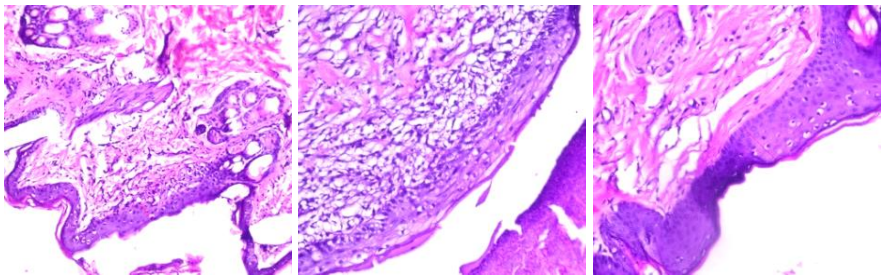

Figure 3. Representative pictures of microscopic features of full thickness wound epithelialization evaluated on day 7 from nanofat-FDHAM combination group.

Epithelization percentage

On day 3, both groups exhibited a median epithelialization percentage of 0%. By day 7, the experimental group demonstrated a slight increase to 0.1%, while the control group remained at 0%. The Mann-Whitney test indicated no significant difference between the groups on day 3 (p=1.000) and approached significance on day 7 (p=0.060) (**Table 1**).

Epidermal growth factor (EGF) levels

EGF levels were significantly elevated in the FDHAM group on both assessment days (**Table 1**). On day 3, the FDHAM group had a median EGF level of 367,826 pg/mL, compared to 273,913 pg/mL in the nanofat-FDHAM combination group (p<0.001). On day 7, EGF levels were 108,696 pg/mL in the FDHAM group versus 70,000 pg/mL in nanofat-FDHAM combination group (p<0.001).

Discussion

Wound healing is a complex process involving wound contraction and epithelialization [19]. In animal models, such as rabbits and mice, wound contraction is the predominant mechanism, whereas epithelialization is more pronounced in humans [20]. In this study, both macroscopic and microscopic assessments on day 3 indicated an absence of epithelial cells in either group, aligning with the typical characteristics of the early inflammatory phase, during which epithelialization has not yet been initiated. By day 7, the experimental group (receiving FDHAM with nanofat) exhibited increased epithelialization, in contrast to the control group (receiving FDHAM alone), which showed no significant changes. These findings suggest that the combination therapy may enhance the regeneration of epithelial cells, facilitating a transition beyond the initial inflammatory phase.

In the present study, the initial raw surface areas of the wounds were comparable for both groups on day 3. However, by day 7, a significant reduction in wound surface area was observed in the experimental group, suggesting that the combination of nanofat and FDHAM promoted more effective wound closure. This finding indicates that epithelialization in full-thickness wounds may require additional time, and the synergistic effects of nanofat with FDHAM could enhance healing, although the full therapeutic effects may not be apparent during the initial evaluation period.

The methodology for fat harvesting in the present study utilized block excision, in contrast to the liposuction technique with a standard cannula employed by Xu *et al*. [13]. Both studies administered the same dosage of nanofat (0.2 mL); however, its application methods differed. The findings of the present study align with those of Foubert *et al*. [22], who observed that both local injection and topical spray could similarly influence inflammation, angiogenesis, and epithelialization [22]. This suggests that although the mode of administration may vary, the presence of nanofat itself plays a significant role in promoting wound healing [22].

The increased levels of EGF observed in the experimental group in the present study on days 3 and 7 ($p<0.001$) underscore the potential of nanofat to significantly enhance growth factor levels compared to FDHAM alone. The EGF levels in the experimental group ranged from 396,522 to 573,913 pg/mL, which are markedly higher than the values reported for FDHAM, which ranged from 71,625 to 109,869 pg/g tissue. This substantial increase is likely attributable to the rich content of EGF and other growth factors present in nanofat, which may promote epithelial cell migration, adhesion, differentiation, and inhibit apoptosis, thereby facilitating more effective epithelialization [23-25].

The present study has several limitations. Firstly, the short evaluation period may not adequately capture the long-term benefits of combining nanofat with FDHAM for complete epithelialization in full-thickness wounds. Additionally, the limited sample size and the use of a single animal model may restrict the generalizability of the findings to human wounds. The present study also did not address potential side effects or long-term outcomes associated with using nanofat in wound healing. Future research should aim to extend the evaluation period to better assess the long-term effects and efficacy of nanofat in combination with FDHAM. Investigating additional animal models and conducting clinical trials in humans could provide more comprehensive insights into the benefits and potential drawbacks of this combination therapy. Furthermore, subsequent studies should explore the optimal dosage and administration methods, as well as the impact of nanofat on various types of wounds and patient populations.

Conclusion

Combining nanofat with freeze-dried human amniotic membrane did not significantly accelerate epithelialization of full-thickness wounds by day 7. However, this combination notably increased EGF levels in the wounds, suggesting that nanofat may enhance the wound's biological environment, although it did not lead to faster epithelialization within the initial seven days.

Ethics approval

The protocol of the present study was reviewed and approved by the Ethical Committee, Faculty of Dental Medicine Health Research, Universitas Airlangga, Surabaya, Indonesia (Approval Number: 1352/HRECC.FODM/XII/2023).

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None to declare.

Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

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

How to cite

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