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Comparison of freeze dried human amniotic membrane and its combination with platelet rich fibrin for the epithelial acceleration in an acute wound excision using *Rattus Novergicus* study model

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ABSTRACT

Introduction: Human amniotic membrane and platelet rich fibrin (PRF) are biological materials that can accelerate wound healing through their content of growth factors and cytokines. This study aimed to determine the effectiveness of the combination of these two biological agents for wound healing.

Methods: This study used an experimental method with a randomized post-test only control group design, to compare the use of human amniotic membrane alone with a combination of human amniotic membrane and PRF in acute full thickness wounds of the skin of Wistar rats. The research subjects were 32 rats which were divided into four groups. Groups 1 and 2 used human amniotic membrane, while groups 3 and 4 used a combination of human amniotic membrane and PRF, then the epithelialization process was observed on the 3rd and 6th day.

Result: There was no statistically significant difference in the speed of epithelialization of excision wounds on the backs of rats on the third day (Significance 0.498) and Sixth (Significance 0.9) between the Amnion group and the combination of amnion and PRF because it was greater than $\alpha = 0.05$.

Conclusion: The use of a combination of freeze dried human amniotic membrane and platelet rich fibrin was not significantly more effective than freeze dried human amniotic membrane in accelerating the epithelialization of acute full thickness excision wounds in rats (Rattus novergicus).

Keywords: human amniotic membrane, platelet rich fibrin, wound healing, immunology.
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INTRODUCTION

Wound is a state of disruption of the integrity of tissues or cells due to a cause, such as damage to the skin layer, tearing of muscles, broken bones or due to heat trauma. Wounds can occur for various reasons, they can be physical, chemical, heat, microbial or due to immune processes. Dead or damaged tissue will be replaced by new tissue through a process called the wound healing process. While wound healing is the process of returning the wound to its original shape, by reducing the wound area to its original size. Wound healing methods have developed in recent years, one of which is the role of the Human Amniotic Membrane and Platelet Rich Fibrin (PRF) gel. The amniotic membrane is the inner layer of the fetal membrane (the outer layer formed by the chorion) and has been studied as an alternative biomaterial for various purposes in reconstructive surgery and wound healing research since it was first introduced as a transplant material by Davis in 1910. The amniotic membrane is rich in collagen and produces amniotic fluid in the early stages of pregnancy. In addition to accelerating wound healing, amnion membranes are also able to reduce pain, reduce the degree of infection and reduce surface fluid loss in burns. In another study in 1982, amniotic membranes were used to cover facial dermabrasion vulnus in 33 patients. The results were "very good" and demonstrated the "advantages of the amniotic membrane over other wound dressing techniques used".[1-4]

Platelet-rich fibrin was first developed as an autologous leukocyte- and platelet-rich fibrin (L-PRF) biomaterial in France since 2001.[5] Unlike other platelet-rich products, this technique does not require anticoagulants or bovine thrombin or other gelling agents. Thus, this fibrin rich platelet is considered as a second-generation platelet concentrate.[6] Platelet-rich fibrin (PRF) is an immune concentrate and platelets aggregate in a fibrin membrane that contains all the elements of the blood sample that support healing and immunity. The material is a rich source of growth factors and its application has been reported as an effective means of inducing tissue response and regeneration. There are at least 60 different biologically active

substances present in platelets that are involved in tissue repair mechanisms such as chemotaxis, cell proliferation and differentiation, angiogenesis, intracellular matrix deposition, immune modulation, antimicrobial activity, and remodeling. Studies have shown that PRF supports three key wound healing mechanisms namely angiogenesis, immunity and epithelial proliferation and thus has implicated its use to protect open wounds and promote healing.[5] In a study conducted by Sari et al (2019), stated that fibroblast levels obtained in treatment with PRF increased very quickly in the first 24 hours compared to treatment with amnion. PRF can stimulate fibroblast proliferation within the initial 24 hours, but the amniotic membrane can duplicate the number of fibroblasts within the next 24 hours.[7] The amnion and PRF also contain the cytokine IL-6 which has been shown to increase keratinocyte proliferation and migration, and indeed, IL-6 deficiency has a significant delay in re-epithelialization in mouse wounds.[8-11]

Based on some of the literature above, researchers are interested in comparing the effectiveness of using human amniotic membrane and its combination with PRF for acute wound care and then observing the speed of epithelialization in the resulting wound healing. Therefore, this study aimed to determine the effectiveness of the combination of these two biological agents for wound healing. It is hoped that the research results can be the basis for developing the use of platelet rich fibrin gel combined with human amniotic membrane as a component of advanced therapy in extensive and mass wound healing with minimal scarring. Soetomo Surabaya can produce a useful product that has better quality.

METHODS

This study used an experimental research design, randomized post-test only control group design. The subject of this study was a full thickness acute excision wound with a size of 2x2 cm^2 which was made on the back of Rattus novergicus strain Wistar. The research subjects were divided into four groups, namely: the group of acute excision wounds on rats which were given treatment using freeze dried human amniotic membrane up to D-3, the group of acute excision wounds on rats which were given treatment using freeze dried human amniotic membrane up to D-6, the group of acute excision wounds in mice treated with freeze dried human amniotic membrane + PRF up to D-3, and the group of acute excision wounds in rats treated with freeze dried human amniotic membrane + PRF up to D-6.

The sample size in each sample group was determined using the formula and obtained for each sample were 6 rats, so there were 24 rats used in this study. To avoid the possibility of the experimental animal dying, (f) = 10%, then the number of replications is multiplied by 1/1-f so that: 1/(1- 0.1) x 6 = 8, with the consideration that a minimum normal distribution is achieved in a sample with a total of 30, So in this study each group used 8 mice, so the number of replications in this study was 8 mice per sample and a total of 32 mice were used. The number of samples available is added to the number of experimental animals needed to take blood alone, amounting to 5, so the total experimental animals needed are 37 animals.

The research variables that will be examined in this study consist of independent variables and dependent variables. The independent variable in this study was obtained from human amniotic membrane and human amniotic membrane + PRF, while the dependent variable in this study was the speed of epithelialization on D-3 and D-6.

To calculate the percentage test for the difference in speed of decreasing wound area between the amnion group and the amnion + PRF group on day 3 or day 6 is obtained by using the formula:

$$\%\ \text{Wound area} = \frac{\text{Wound area of zero day} - \text{wound area either of third day or sixth day}}{\text{Wound area of zero day}} \times 100\%$$

to calculate the difference in speed of decrease in wound area (mm^2/day) between the amnion group and the amnion + PRF group on day 3 or day 6 was obtained using the formula:

$$\text{Epithelization acceleration } (mm^2/\text{days}) = \frac{\text{Wound area of zero day} - \text{wound area either of third day or sixth day } (mm^2)}{\text{time (days)}}$$

The research data was taken and collected according to each treatment group, namely: in the treatment group 1 the incision wound on the rat's back was closed using a human amniotic membrane, in the 2nd treatment group the incision wound on the rat's back was closed using a human amniotic membrane + PRF, in this way stick the HAM which has been made three holes with a distance of 0.2 cm and 1 mm wide, then PRF is smeared on the superficial side of the amnion so that PRF can contact the wound surface through the holes in the amnion and on top of it is covered with thick gauze. Measurements were carried out by three reviewers using the images obtained which were copied to digital visitrax to determine the area of the area that had not been covered by the epithelium and the results of the three reviewers were averaged so that the speed of epithelialization of each subject was obtained. Existing data were collected to be analyzed with blood to find out whether there were significant differences in wound area and speed of epithelialization in each using a statistically different test.

RESULT

Table 1 shows a description of the study subjects who met the inclusion and exclusion criteria. Each experimental animal has an acute full-thickness excision wound which is then measured by measuring the area of the wound using transparent plastic, then proceed with wound care according to the results of

Table 1. Description of research subjects

Characteristics	HAM	HAM + PRF
Average age (month)	3	3
Gender		
Male	16	16
Female	-	-
Location		
Mouse back	16	16

HAM: Human Amniotic Membrane; PRF: Platelet Rich Fibrin

Table 2. Data on wound area and delta area on two treatments with HAM and PRF (in cm²)

Number	Wound area					
	3rd day		6th day		Delta in 3rd and 6th day	
	Amnion	Amnion + PRF	Amnion	Amnion + PRF	Amnion	Amnion + PRF
1	1.8	3.5	1.2	1.1	-0.6	-.24
2	3.4	2.1	1.9	1.2	-1.5	-0.9
3	2.3	3.7	1.4	2.9	-0.9	-0.8
4	2.3	2.7	1.9	1.4	-0.4	-1.3
5	2.8	3.4	1.4	1.9	-1.4	-1.5
6	3.5	2.7	1.9	2.4	-1.6	-0.3
7	2.9	3.2	1.8	2.4	-1.1	-0.8
8	4	3.5	2.7	1.2	-1.3	-2.3
Mean	2.88	3.10	1.78	1.81	-1.10	-1.29
SD	0.73	0.55	0.47	0.69	0.43	0.75
P value	0.498		0.900		0.549	

Notes:

Abbreviations: HAM, Human Amniotic Membrane; PRF, Platelet Rich Fibrin; SD, Standard Deviation

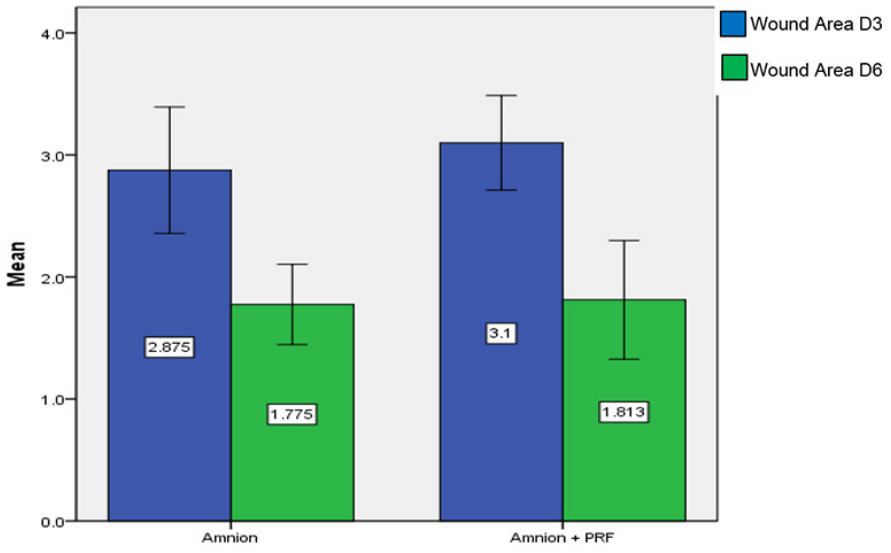

Figure 1. Graph of Amnion and Amnion Research Results + PRF

randomization, namely by using a human amniotic membrane or using a combination of PRF + human amniotic membrane. After being given treatment, the wound is then wrapped with a transparent dressing, thick gauze and pressure dressing. The measurement results are then copied into the grid visitrak, which is then converted in cm^2 area units using a digital visitrak. This measurement was carried out 2 (two) times, namely on the 3rd day and 6th day. On the 0th day measurement, the excision wound was measured which was taken as the subject, assessed in % units (on the 0th day it was considered 100%). On the 3rd and 6th day, wound measurements were taken again in % units. The percentage of the wound area epithelialization process is obtained by calculation.

Table 2 and Figure 1 show that treatment with human amniotic membrane resulted in a smaller wound area on D-3 and D-6 compared to human amniotic membrane + PRF. The combination of freeze dried human amniotic membrane + Platelet Rich Fibrin was not significant in accelerating the epithelialization of acute full thickness excision wounds than the use of freeze dried human amniotic membrane. The average H-3 wound area in the Amnion group was 2.88 + 0.73 lower than the average H-3 wound area in the Amnion + PRF group, which was 3.10 + 0.55. The results of testing the difference with the independent t test yielded test statistics on amnion and amnion + PRF on D-3 obtained a significance value greater than $\alpha = 0.05$, which is 0.498. Therefore, it can be stated that statistically there is no significant difference in the area of back excision wounds of H-3 rats between the Amnion and Amnion + PRF groups. Then the H-6 wound area in the Amnion + PRF group was 1.81 + 0.69 which was higher than the average H-6 wound area in the Amnion group which was 1.78 + 0.47. The results of testing the difference with the independent t test yielded test statistics on the amnion and amnion + PRF on the sixth day obtained a significance value greater than $\alpha = 0.05$, which is 0.900. Therefore, it can be stated that statistically there is no significant difference in the area of back excision wounds of H-6 rats between the Amnion and Amnion + PRF groups.

The percentage decrease in wound

area can be seen in Table 3 and Figure 2 in the back excision wounds of rats from day 0 to day 3 treated using human amniotic membrane, the results obtained were 28.13%, with a standard deviation of ± 18.31, and the percentage decrease in wound area. In the back excision wounds of rats from day 0 to day 3 treated using human amniotic membrane + PRF, the yield was 22.50%, with a standard deviation of ± 13.69. The p-value for the percentage decrease in wound area in the back excision wounds of rats from day 0 to day 3 was 0.498 ($p > 0.05$) indicating that there was no significant difference between the percentage reduction in wound area in the back excision wounds of rats that received treatment with human amniotic membrane and human amniotic membrane + PRF. The percentage decrease in wound area in the back excision wounds of rats from day 0 to day 6 treated using human amniotic membrane obtained results of 55.63%, with a standard deviation of ± 11.63, and the percentage decrease in wound area in back excision wounds of rats from day to day -0 to the 6th day treated using human amniotic membrane + PRF obtained a result of 54.69%, with a standard deviation of ± 17.19. The p-value for the percentage of wound area in the back excision wounds of rats from day 0 to day 6 was 0.900 ($p > 0.05$) indicating that there was no significant difference between the percentage reduction in the area of the wound area in the back excision wounds of rats that received treatment with human amniotic membrane and human amniotic membrane + PRF.

Table 3. Description of research data on the decrease in wound area (in %)

Number	Percentage of wound area					
	3rd day		6th day		Delta in 3rd and 6th day	
	Amnion	Amnion + PRF	Amnion	Amnion + PRF	Amnion	Amnion + PRF
1	55	12.5	70	72.5	15	60
2	15	47.5	52.5	70	37.5	22.5
3	42.5	7.5	65	27.5	22.5	20
4	42.5	32.5	52.5	65	10	32.5
5	30	15	65	52.5	35	37.5
6	12.5	32.5	52.5	40	40	7.5
7	27.5	20	55	40	27.5	20
8	0	12.5	32.5	70	32.5	57.5
Mean	28.13	22.50	55.63	54.69	27.50	32.19
SD	18.31	13.69	11.63	17.19	10.86	18.68
P value	0.498		0.900		0.549	

HAM: Human Amniotic Membrane; PRF: Platelet Rich Fibrin; SD: Standard Deviation

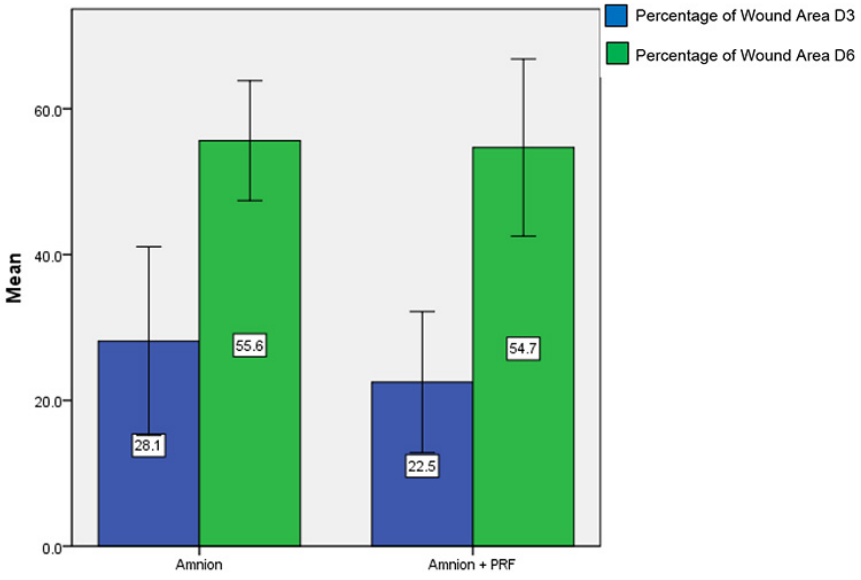

Figure 2. Graph of Research Results Percentage of Amniotic Wounds and Amnion + PRF

Based on Table 4 and figure 3, it shows the speed of decrease in wound area obtained in the back excision wounds of rats treated using human amniotic membrane from day 0 to day 3 was 37.5 mm^2/day, with a standard deviation of ± 24.41 mm^2/day , and the speed of reduction in the area of the back excision wound in rats treated with human amniotic membrane + PRF from day 0 to day 3 obtained results of 30.00 mm^2/day, with a standard deviation of ± 18.26 mm^2/day. The speed of reduction in wound area on day 3 obtained a p-value of 0.497 ($p > 0.05$), so it was said that there was no significant difference in the speed of reduction in the area of back wounds of rats treated with human amniotic membrane and human amniotic membrane + PRF from day 0 to day 3. The speed of reduction in wound area was found in the back excision wounds of rats treated using human amniotic membrane from day 0 to day 6 of 37.08 mm^2/day, with a standard deviation of ± 7.75 mm^2/day, and the speed of reduction in the area of excision wounds the backs of rats treated using human amniotic membrane + PRF from day 0 to day 6 obtained results of 36.46 mm^2/day, with a standard deviation of ± 11.46 mm^2/day. The speed of reduction in wound area on the 6th day obtained a p value of 0.900 ($p > 0.05$), so it was said that there was no significant difference in the speed of reduction in the area of back wound of rats

Tabel 4. Description of the results of the research on the acceleration of epithelialization (mm²/days).

Number	Epithelialization acceleration					
	3rd day		6th day		Delta in 3rd and 6th day	
	Amnion	Amnion + PRF	Amnion	Amnion + PRF	Amnion	Amnion + PRF
1	73.3	16.7	46.7	48.3	-26.7	31.7
2	20.0	63.3	35.0	46.7	15.0	-16.7
3	56.7	10.0	43.3	18.3	-13.3	8.3
4	56.7	43.3	35.0	43.3	-21.7	0.0
5	40.0	20.0	43.3	35.0	3.3	15.0
6	16.7	43.3	35.0	26.7	18.3	-16.7
7	36.7	26.7	36.7	26.7	0.0	0.0
8	0.0	16.7	21.7	46.7	21.7	30.0
Mean	37.50	30.00	37.08	36.46	-0.42	6.46
SD	24.41	18.26	7.75	11.46	18.51	18.59
P value	0.498		0.900		0.549	

treated with human amniotic membrane and human amniotic membrane + PRF from day 0 to day 6.

DISCUSSION

PRF is a platelet concentrate consisting of PDGF, TGF-β, including β-1 and β-2 isomers, VEGF, and EGF, all of which are capable of maintaining skin viability.[12] Dohan et al (2006) have reported on fibrin glue and fibrin, PRP concentrate, and PRF.[5] All three types of fibrin are highly dependent on artificial polymerization, such as the use of massive bovine thrombin. PRF undergoes slow polymerization of fibrin in PRP, causing the structure of PRF to resemble that of natural fibrin, which contributes to cell migration, cell proliferation, and cicatrix formation.[13,14] In laboratory experiments using special media for PRF, levels of PDGF, VEGF, FGF, and TGF-β increased on the first day and then decreased gradually the next day. PRF has fibrin characteristics with a platelet distribution that more closely resembles the body's response to injury and a twisted macroscopic structure.[15]

The amnion is used as a biological dressing in cases of burns (superficial burns), as well as a covering for donor split-thickness skin grafts. The advantages of using amnion compared to other biological skin substitutes are due to its low antigenicity, antimicrobial properties, and ability to reduce exudate and adhesions, accelerate epithelialization, reduce local pain, and act as a substrate for tissue growth.[16]

In an experimental study conducted on the back excision wounds of H-3 rats between the Amnion and Amnion + PRF groups, the significance value was greater than α = 0.05, namely 0.498; which concluded that statistically there was no significant difference. The area of the H-6 wound in the Amnion + PRF group produced statistics with a significance value greater than α = 0.05, namely 0.900; so it can be stated that there was no significant difference. The percentage of epithelial velocity or decrease in wound area was calculated after the wound area was measured and obtained from the difference between the wound area of the rat's back on day 0 and the wound area on day 3 or 6. The results of the calculations show that there is no significant difference between the percentage reduction in wound area in the back excision wounds of rats that received treatment with human amniotic membrane (HAM) and human amniotic membrane + PRF on D-3 and D-6.

The results of the above study showed that there was no significant difference in both the wound area and the speed at which the wound area decreased on D-3 and D-6. The results of the same study on PRF were also shown by one study, on skin graft donors with the result that there was no significant difference between PRF and the control group regarding epithelial closure of the donor wound on the 5th and 8th day. However, different results were shown in a study conducted by Vaheb et al in 2021 where the average wound healing time in PRF was significantly lower than the control group ($P < 0.001$). The PRF group showed significantly higher wound healing rates than the control group at 8 and 15 days ($P < .001$ and $P < .001$, respectively).[17] These different results may be due to differences in the method of application of PRF where in a study conducted by Vaheb et al[17] all PRF was rubbed evenly over the entire surface of the wound so that contact with the wound was maximized, but in a study carried out a combination of PRF + HAM is done by sticking the HAM in which 3 holes have been made with a distance of 0.2 cm and 1 mm wide, then applying PRF on the superficial side of the freeze dried human amnion membrane so that the PRF can contact the wound surface through the holes.

CONCLUSION

The use of a combination of freeze dried human amniotic membrane and platelet rich fibrin was not significantly more effective than freeze dried human amniotic membrane in accelerating the epithelialization of acute full thickness excision wounds in rats (Rattus novergicus).

CONFLICT OF INTEREST

The author reports no conflicts of interest in this work.

FUNDING

None to declare.

AUTHOR CONTRIBUTION

All authors contributed equally in the research.

ETHICAL STATEMENT

This study has obtained approval from the Animal Care and Use Committee (ACUC) No.2.KE.193.11.2019

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