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Topical hydrogel human amnion membrane for wounds healing in mice (mus musculus) induced by diabetes

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Abstract

Background: One of the complications that occurs in DM patients is the occurrence of chronic wounds. Human Amnion Membrane (hAM) has been identified as a promising natural option for treating skin lesions, including burns and diabetes mellitus. This hAM contains extracellular matrices such as growth factors, collagen, and proteoglycans as anti-inflammatory and antimicrobial agents in wounds.
Objective: to analyse the effect of hAM hydrogel treatment on wound closure macroscopically and microscopically on days 3, 7, 14, and 21.
Methods: include hydrogel characterisation tests using SEM and ATR-FTIR. Experimental procedures, using mice divided into 3 treatment groups, namely healthy and DM groups without treatment, and hamd DM treatment. Wound area measurements (macroscopic) and hematoxylin-eosin staining were carried out. Two-way ANOVA test analysis using Graphpad Prism 10.
Results: The healthy group without treatment and the DM group with hAM treatment on day 21 had closed wound closure, whereas the DM group without therapy on day 21 had no closed wound closure. The above results align with the statistical and histopathological analysis results.
Conclusion: Human amniotic membrane hydrogel administered to DM mice's skin wounds can heal diabetic skin wounds faster than the control group, namely healthy mice and DM mice without treatment.

Keywords: Diabetes mellitus, hydrogel, extracellular matrix, amniotic membrane

INTRODUCTION

Wounds are a form of tissue damage to the skin from the simplest such as the epithelial layer of the skin, to deeper layers, namely subcutaneous tissue, fat, muscle and even bone with other structures due to trauma to tendons, blood vessels, muscles and nerves. Chronic wounds caused by diabetes take a long time to heal because they fail to regenerate naturally and often heal with the formation of fibrotic tissue with impaired structure and mechanical properties. Inappropriate wound care has the potential to cause more extensive tissue damage and can result in a prolonged wound healing process. In high glucose conditions as a result of diabetes, skin elasticity appears impaired and damages the secretion of extracellular matrix materials, so that the ability to migrate and grow cells decreases due to a lack of growth factors and extracellular matrix (ECM) components(1).

The number of diabetics is increasing from year to year, which can cause an increase in diabetic complications, including diabetic ulcers. Diabetic wounds are caused by infection from high blood glucose levels, resulting in increased bacterial proliferation and immune system deficiency. It causes the inflammatory process in healing, which takes a long time and causes persistent infections that will lead to amputation if left untreated (2).

Treatments to accelerate the healing process of diabetic wounds include replacing damaged tissue with biocompatible substitutes such as autografts, allografts, and xenografts. However, all three options have disadvantages, including the wound healing process taking longer, causing new

wounds, complications of infection and requiring high costs (3).

Treatment in powder form has limitations, namely, it is applied to the wound by sprinkling it so that this product only heals external wounds (3). And there is treatment in ointment preparations, but it has a sticky and slightly oily texture. Along with advancing technology and science, many alternatives have been developed to treat wounds, including wound dressings using hydrogel (4). One of the materials that can accelerate wound healing is ECM. One of the materials containing ECM is known to be biocompatible and biodegradable, namely Human amnion membrane (hAM).

HAM is an extracellular matrix containing collagen derived from the human placenta. HAM includes a lot of ECM to reduce inflammation and the appearance of scar tissue, increase epithelialization and wound healing, and have antibacterial effects. In the study by Pakpahan et al., 2024, it was stated that in test animals with DM, when treated using HAMD, wound closure was faster compared to the group of healthy and DM mice that were not given treatment, because HAMD has ECM and can synthesize collagen well (5).The amniotic membrane also contains biologically active substances, namely angiogenic substances that form granulation tissue in wound healing (6). Although HAM is included in medical waste, HAM is biocompatible because it has extraordinary structural and regenerative components, especially as a skin dressing, due to its extracellular matrix structure and excellent wound healing biological properties. HAM contains several cells, including epithelial cells, fibroblasts, and mesenchymal stem cells.

METHOD

This type of research is quantitative research. The design used in this study is an experimental study using mice divided into 4 treatment groups, namely healthy group non treatment, DM non treatment), and DM treatment hAM. Data collection techniques in this study used primary data. Primary data were collected from direct observation results by measuring the area of the wound, by measuring the area of the back wound of mice using a caliper and microscopic observation of mouse skin tissue preparations on days 3, 7, 14 and 21 using an Olympus CX300 microscope. Data were analyzed using the Two-way ANOVA statistical test using Graphpad Prism 10.

Tools And Materials

The tools used in this study include glucometer (Nesco), spatula, scalpel, tweezers (One Med), 1cc syringe (Stera), analytical balance (Excellent), jar, magnetic stirrer, 100ml beaker (Iwaki), Olympus CX33 microscope. Then the materials used include alloxan (Sigma Aldrich), distilled water, male mice, glucose test strips, 10% NBF, 70% alcohol, parafilm, tissue.

Research Procedure

Methods

1.Preparation of Human Amniotic Membrane

The placenta was placed in a sterile container containing transport media, 1x PBS+4% ABAM solution. Washed using sterile saline solution containing antibiotics under aseptic conditions. Slowly, the amnion layer was separated from the chorion, then the hAM was transferred to a sterile PBS+1% ABAM solution(8).

2. Cell culture

Human Dermal Fibroblast (HDF) was cultured in DMEM (Sigma-Aldrich, D5796, Germany) containing 10% fetal bovine serum (FBS, Gibco™, 16000044, USA), 1% Antibiotic-Antimycotic (ABAM, Sigma-Aldrich, A5955, Germany). The medium was changed every 48 hours.

3. hAM Characterization

Scanning Electron Microscopy (SEM) was used to observe the morphology of HDF cells in hAM. The samples were fixed with 100 µL of 2.5 glutaraldehyde in 0.1 M cacodylate buffer, incubated overnight at 4°C, dried with an alcohol series, and dried with HMDS overnight. The samples were coated with gold and observed under SEM (SU 3500; Hitachi, Krefeld, Germany).

Cell staining using DAPI. Cells in the well plate were rinsed with Phosphate Buffer

Saline (PBS) (Sigma-Aldrich, D8537, Germany) (shaking) and then fixed with methanol-DMEM and methanol-PBS (50%:50%). Cells were placed on a glass slide and then visualized with a confocal microscope (Confocal Laser Scanning Microscope Olympus FV-1200).

Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy was performed using the FTIR Broker Alpha II, between 1000cm−1 and 4000cm−1. The working principle of ATR-FTIR is to measure the changes that occur in the process of infrared light reflection when the light approaches the sample.(9)

4. Preparation of Test Animals

The preparation of test animals used is healthy mice weighing around 20-35 grams with an age of 2-3 months. Then the mice are adapted for 7 days to adjust to the new environment and are fed and given water twice a day.

5. Alloxan Induction

According to Hilmi (2021) before being induced with alloxan, blood sugar levels were checked by cleaning the tip of the mouse's tail using 70% alcohol and then piercing the tip of the mouse's tail using a sterile lancet needle, then a drop of blood was dripped onto a sugar strip to read the glucose level. Alloxan solution was induced intraperitoneally as much as 0.5cc using a 1cc syringe. Mice were given food and drink as usual after injection. Then stabilized for 7 days after induction with alloxan solution. Mice that have blood sugar levels of 200mg/dl can be said to be hyperglycemic and further experiments can be carried out.

6. Wound Making

Wound making is done by anesthetizing the mouse using cotton that has been dripped with chloroform until the mouse is limp. After that, shave the mouse's back fur and then make a wound measuring 1x1 cm until it reaches the dermis which is marked by the release of blood.

7. Application of 15% hAMD Hydrogel

The application of 15% hAMD hydrogel was carried out using a one-way technique of <0.1 grams using a cotton bud 3 times a day, namely in the morning at 06.00 WIB, afternoon at 14.00, and evening at 22.00 WIB consisting of 3 treatment groups on days 3, 7, 14, and 21. Furthermore, the area of the back wound was measured using a caliper every day at 22.00 WIB.

8. Tissue Collection

According to Fadillah et al., (2023) tissue collection is carried out by anesthetizing mice using chloroform by inhalation until the mice do not move, then shaving the growing fur. Then the mouse skin tissue is taken with a size of 2x2 cm up to the subcutaneous, then the tissue is inserted into a cassette and labeled and fixed with 10% NBF for 24 hours.

9. Tissue Processing

The process of making histopathology preparations is a sample that has been fixed using a 10% NBF solution, then dehydrated using graded alcohol, then do the clearing process using xylol. The next stage is the blocking stage where the tissue is inserted into the base mold and filled with paraffin. The tissue block is sectioned with a thickness of 5um using a microtome, the ribbon cut results are stretched over warm water at a temperature of 46 ° C and immediately lifted so that it does not fold. The preparation is lifted and placed on a glass object and dried overnight in an incubator at a temperature of 60°C.

10. Hematoxylin Eosin (HE) Staining

Hematoxylin eosin staining begins with the deparaffinization stage using xylol to remove paraffin, followed by the rehydration stage using alcohol of decreasing concentration, then hematoxylin staining. After hematoxylin staining, the tissue preparation is washed with running water and enters the decolorization stage using acid alcohol followed by washing with water again. The next stage is bluing using lithium carbonate to clarify the blue color in the cell nucleus, eosin staining, dehydration with alcohol of increasing concentration and clearing using xylol. After the staining is complete, the last stage is mounting using entelan to cover the tissue preparation.

11.Qualitative Measurement (Histology Preparation)

In this study, observations were made on histology preparations of skin tissue with Hematoxylin Eosin staining using an Olympus CX33 microscope at 400x magnification.

12. Quantitative Measurement

In this study, the number of PMN, MN, fibroblasts and angiogenesis cells of histological preparations of skin tissue were calculated with Hematoxylin Eosin staining using an Olympus CX33 microscope at 400x magnification.

13. Data Analysis

The data obtained in this study were the results of macroscopic observations, namely the size of mouse wounds and microscopic observations, namely the number of PMN, MN, angiogenesis, and fibroblasts. Data were analyzed using the Two-way ANOVA statistical test using Graphpad Prism 10.

RESULTS

1. hAM Characterization

a. Scanning Electron Microscope (SEM)

Figure 1B shows the SEM results of hAM used with cells. The cells show substantial accumulation or clustering due to the persistence of epithelial cells on the hAM substrate. The SEM data also show the presence of fibroblast cells, which are marked with red arrows.

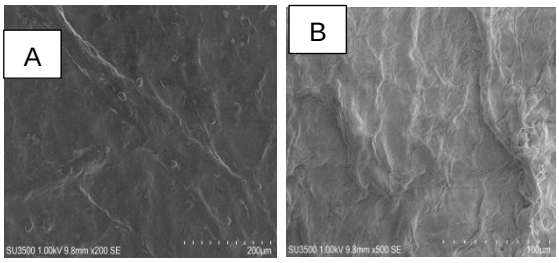

Figure 1. Morphology of hAM: (A) fresh hAM without cells; (B) fresh hAM using cells; Presence of fibroblast cells. Magnification: 500x.

b. Confocal DAPI

Confocal microscopy was used to visualize the presence of core residues in hAM through DAPI labeling. In Figure 2A, freshly seeded hAM showed high cell density, indicating potential overlap between epithelial cells derived from decellularized hAM and seeded human dermal fibroblast (HDF) cells. The data presented in Figure 2B illustrate the 3-dimensional DAPI staining observed from top and side perspectives. It is clear that the cells are centrally positioned.

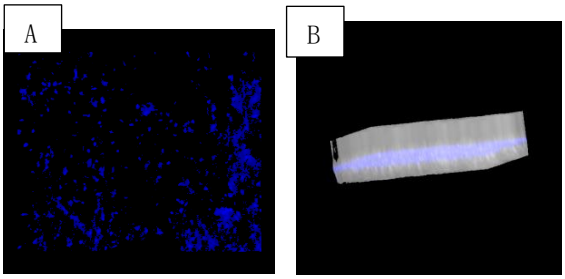

Figure 2. DAPI staining results. (A) 3D visualization of fresh hAM. (B) 3D visualization of fresh hAM viewed from the side and top, viewed from above.

c. ATR-FTIR

ATR-FTIR analysis of decellularized hAM at certain wave numbers, such as 1652 cm-1, 1550 cm-1, 1339 cm-1, and 1200 cm-1 (Figure 3).

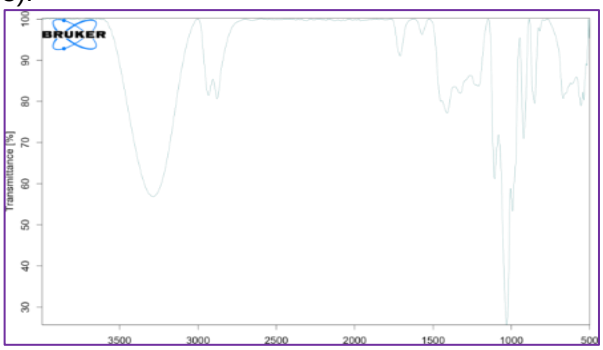

Figure 3. ATR-FTIR characterization; hAM hydrogel.

2. Mouse Wound Closure

Preliminary tests were conducted on healthy mice and untreated DM mice. In the healthy mice group, on the 21st day the wound had closed completely, but in the DM mice group, on the 21st day the wound had not closed completely.

In Figure 4, it can be seen that there is a decrease in the wound area from day 0, day 3, day 7, day 14, to day 21. The wound closure of mice treated with carbopol hydrogel and hAMD hydrogel closed 15% faster, while for mice not treated, the wound closure was slower. The size of the wound area was analyzed statistically using GraphPad Prism 10 software which can be seen in Figure 5.

Day-3 Day-7 Day-14 Day-21

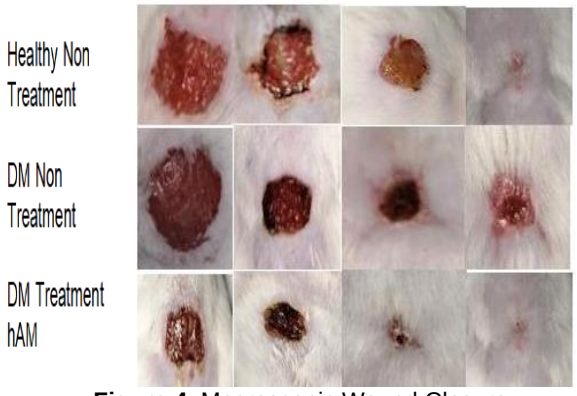

Figure 4. Macroscopic Wound Closure

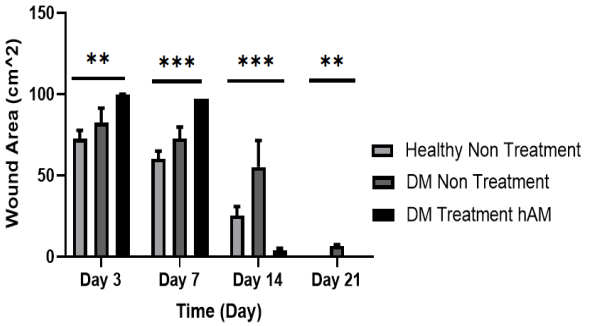

Figure 5. Wound closure in healthy mice group Non-treated DM mice, DM mice without treatment, DM mice group treated, and DM mice group hAMD. Symbol (***) indicates significant difference (p<0.05).

Tabel 1. Two-Way ANOVA Statistical Test for Wound Closure Results

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	11.73	<.001	***	Yes
Time (Day)	83.58	<.001	***	Yes
Wound Area	2.754	<.001	***	Yes

Based on Figure 5, the analysis of the size of the wound closure area shows that on day 0 and day 3 of the healthy group without treatment, there was wound closure with a significance of p ≤ 0.001. The effect of wound closure between day 0 and day 7 for healthy individuals without treatment with carbopol and those without DM treatment had a significance value> 0.05. The effect of wound closure on day 7 and day 14 between healthy individuals without treatment and DM treatment, carbopol and DM treatment hAMD, has a significance value of p ≤ 0.001, and between DM without treatment and DM treatment hAMD has a significance value of p ≤ 0.001.

The skin wound tissue was stained with hematoxylin-eosin to see the wound closure macroscopically. The results of macroscopic observations can be seen in Figure 6.

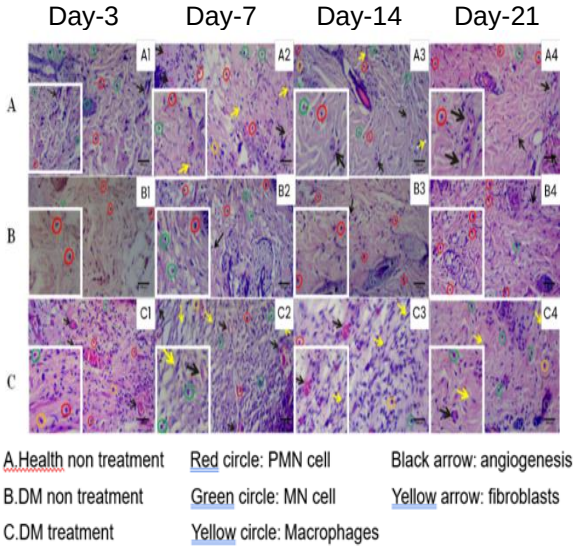

Figure 6. Wound closure in the group of healthy mice without treatment, DM mice without treatment and the group of DM mice treated with the group of DM hAMD mice (microscopic).

Based on Figure 6, it shows the presence of inflammatory cells in each group on day 3. On day 7, fibroblasts were seen and angiogenesis began to form. On day 14, in the healthy group, and DM hAM, the epithelium was already thickened, but for the DM group without treatment, the epithelium was thinner. On day 21, the epithelium in the healthy mouse group was thicker than the DM group non treatment.

DISCUSSION

Diabetic wounds often have a slow healing process and are easily infected due to hyperglycemia at the wound bed. Thus, treatments to accelerate wound healing in diabetic patients are ongoing. One strategy involves the utilization of hAM combined with hydrogel. HAM is the inner layer of the placental membrane. It is a thin, semitransparent membrane that typically ranges from 0.2 to 0.5 mm in tissue thickness (7). Amniotic membrane has been identified as a suitable dressing for burns in developing countries due to its easy

availability, ease of procurement and sterilization, and cost-effectiveness compared to standard dressings(10).

Based on this study, hAM has functional groups at certain wave numbers, namely 1652 cm-1, 1550 cm-1, 1339 cm-1, and 1200 cm-1. This study shows that the ATR-FTIR results of hAM decellularization have functional groups, namely 1652 cm-1, 1550 cm-1, and 1339 cm-1(11).

This study was conducted using HAMD hydrogel derived from the placenta of mothers giving birth. HAM contains a lot of ECM which plays a role in reducing inflammation, increasing wound epithelialization, has antibacterial properties so it can accelerate wound healing (6). According to research conducted by (Murphy et al., 2017), the use of hydrogel for wound healing showed significant wound closure because hydrogel was effective in maintaining and creating moist wound conditions, thereby accelerating the healing process(12).

Hydrogel can be used to deliver bioactive molecules known to accelerate wound healing, angiogenesis, re-epithelialization and production of new ECM maturation. Hydrogel can synergistically modulate inflammation in diabetic wounds through Reactive Oxygen Species (ROS). Reactive Oxygen Species is a condition caused by increased production of free radicals and decreased antioxidant defense activity(13). In addition, to meet the needs of dressing replacement through hydrogel degradation, the wound healing phase thus achieves optimal skin regeneration(14). In a study conducted by (Barski et al., 2018) hAM was used for the treatment of burns and concluded that it significantly increased wound healing. In addition, hydrogel human amnion membrane provides hydration for wounds and effectively prevents wound contraction(15). The use of hAM hydrogel is very influential in accelerating wound closure and epithelialization because it contains many ECM and growth factors. In a study conducted by (Barski et al., 2018) hAM was used for the treatment of burns and it was concluded that it significantly increased wound healing(4). In addition, the hydrogel human amnion membrane provides hydration to the wound and effectively prevents wound contraction(15).

This research was conducted by adapting the test animals first for one week so that the mice could adjust to their environment so that they would not experience stress during the experiment(15) In this study, adult male mice aged 6 weeks, weighing 20-35 grams and male were used. The use of male mice in this study was because they were not affected by hormones and cycles and their stress levels were not too high like female mice. In addition, the consideration of using mice as test animals was because their life cycle was relatively short, easy to handle, and their anatomical, physiological, and genetic structures were similar to humans(17).Before the treatment was carried out, blood sugar levels were measured first on 4 groups, to ensure that the mice to be used did not have congenital diabetes. Three groups of mice for the diabetes treatment model were induced by alloxan intraperitoneally, then after six days of induction, their blood sugar levels were checked. Mice were said to be diabetic if their blood sugar levels were more than 200 mg/dL(2).

On days 3, 7, 14 and 21 three mice were terminated to take skin tissue and then tissue maturation and hematoxylin eosin staining were performed to see wound closure microscopically. The tissue taken was fixed using 10% NBF for 24 hours. Tissue maturation begins with a dehydration process using graded concentration alcohol, then clearing using xylol to clear the tissue from contamination that could interfere with tissue staining(18). Next is infiltration using paraffin. After the tissue maturation process is carried out, the next stage is the planting of tissue on the base mold and given liquid paraffin. The tissue that has become a tissue block is then continued to the microtomy stage. The microtomy process is carried out to cut the tissue block using a microtome so that a tissue band with a thickness of 5 µm is obtained and then attached to the glass object. The preparations that have been

made are stained with hematoxylin eosin to see the structure and morphology of the tissue.

Hematoxylin eosin staining in this study was carried out to see wound closure microscopically. Hematoxylin eosin staining begins with the deparaffinization stage using xylol to remove paraffin, followed by the rehydration stage using alcohol of decreasing concentration, then hematoxylin staining. After hematoxylin staining, the tissue preparation was washed with running water and entered the decolorization stage using acid alcohol followed by washing with water again. The next stage is bluing using lithium carbonate to clarify the blue color in the cell nucleus, eosin staining, dehydration with alcohol of increasing concentration and clearing using xylol. After the staining is complete, the last stage is mounting using entelan to close the tissue preparation. In the post-analytical stage, observations of the tissue preparation were carried out using a microscope at a magnification of 40x and the data from the wound closure were analyzed statistically using GraphPad Prism 10 software.

The results of wound closure macroscopically can be seen in Figure 6, there was a decrease in wounds in each treatment group from day 3, 7, 14, to day 21. On day 3 in mice that were not given hydrogel treatment, the wound condition was still red because the inflammation process was still occurring. On day 7 in all treatment groups there was a decrease in wound area, but a more significant decrease in wound area was in the DM mice group with hAM treatment. Wound closure on day 14, the size of the wound in DM mice that were not given treatment was still large compared to mice that were given hydrogel hAM treatment. In the group of mice that were given hydrogel hAM treatment on day 14, the wound was almost completely closed. Because in mice that were not given treatment on day 14 there were still quite large wounds, it was continued until day 21.

Based on the results of statistical tests of increased wound closure based on days using GraphPad Prism 10 software, the results obtained on day 0 and day 3 of the healthy group without treatment showed an increase in wound closure with a significance of $p \leq 0.001$ and between the healthy group without treatment and DM without treatment the significance value of $p < 0.05$. While for the and DM hAM mice groups there was no increase in wound closure. On the 7th day, the decrease in wound size of healthy mice without treatment and DM non treatment was faster than DM hAM treatment. However, from day 7 to day 14 there was a very significant decrease in the DM hAM treatment groups with a significance value of $p \leq 0.001$. While for DM without treatment from day 7 to day 14 the increase in wound closure was not significant. So it can be said that there is a significant effect of wound closure in mice given hAM treatment.

The results of the statistical test of wound closure based on treatment obtained healthy group without treatment on day 0 to 3 and day 7 to 14 there was an increase in wound closure with a significance of $p < 0.0001$ while on day 3 to 7 there was no significant increase in wound closure. In the DM group without treatment from day 0 to day 3 and day 7 to day 14 there was an increase in wound closure with a significance of $p < 0.05$ while from day 3 to 7 there was no significant increase in wound closure. In the DM hAM groups from day 0 to day 3 and day 3 to day 7 there was no significant increase in wound closure. While from day 7 to day 14 there was a very significant increase in wound closure with a significance of $p \leq 0.0001$.

Based on the results of microscopic observations, on day 3 for all groups of test animals there were inflammatory cells including lymphocytes and monocytes. In the wound healing process, day 3 is included in the inflammatory phase where the inflammatory phase functions to remove damaged cells and prevent bacterial infections. In the inflammatory phase, monocytes differentiate into macrophages entering the wound. Macrophages play a very important role in wound healing because they function to phagocytize bacteria and damaged tissue into

macrophages that will secrete anti-inflammatory cytokines such as IL-4, IL-10, and IL-13. In the inflammatory phase, these cells that appear will release growth factors and cytokines that will attract cells that play a role in the proliferation phase to the wound site and accelerate angiogenesis(13).

On the 7th day, which is the proliferation stage, in this phase the increase in the number of fibroblasts and angiogenesis plays a role in the formation of granulation tissue. In the healthy group non treatment, and DM hAM there are fibroblast cells and angiogenesis. In the proliferation stage, fibroblasts are the main cells responsible for replacing the temporary fibrin matrix into larger granulation tissue. Fibroblasts will respond to signals from platelets, endothelial cells, and transforming growth factors TGF-β and PDGF. These signals will direct fibroblasts to lay ECM and differentiate to encourage wound contraction. While angiogenesis plays a role in maintaining the continuity of the function of various damaged tissues. In angiogenesis, the formation of new blood vessels comes from capillaries that emerge from small blood vessels around them. In mice in the DM group without treatment, there are still inflammatory cells because in diabetes conditions the inflammatory phase lasts a long time due to improper blood supply.

On the 14th day, the formation of epithelium in the DM hAM mice groups was thicker than on the 7th day. While in the healthy mice group without treatment and DM non treatment, the epithelial cells were still thin. Simultaneously, basal cells in the epithelium will move from the edge of the wound to the wound area and cover the wound area. Because in healthy and DM mice there are still quite large wounds, histological observations were carried out on the 21st day for healthy and DM mice. In healthy and DM mice, there are epithelial cells, but for the healthy mice group without treatment, the epithelial cells are thicker because in macroscopic observation the wound has been completely closed, while for DM mice without treatment, the epithelium is still thin because in macroscopic observation the size of the wound is still large.

Wound healing is a complex and sequential process involving various inflammatory cells, angiogenesis, and tissue remodeling. In chronic wound healing, the balance of various cells that affect wound healing becomes unstable because the inflammatory phase lasts a long time, the blood supply is not right, causing immune response disorders and infections, and the reepithelialization process decreases. The use of hAM for wound healing is because hAM has antimicrobial, antifibrotic, and anti-inflammatory properties. When used for wounds, hAM will easily stick to the wound surface, reduce scars, reduce pain, improve wound healing, and have low immunogenicity. hAM contains a number of angiogenic factors that play a very important role in the wound healing process, including TGF-β and bFGF which affect various types of cells such as fibroblasts and keratinocytes so as to stimulate cell migration and proliferation, increase temporary ECM synthesis, formation of new blood vessels, modulation of collagen deposition, and remodeling of temporary ECM into permanent ECM in granulation tissue(19)(20)(21).

CONCLUSIONS

This study concludes that the effect of human amniotic membrane hydrogel can close diabetes mellitus skin wounds faster compared to healthy mice and non-treated DM groups.

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