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Research Article

The Tissue Structure in the Remodeling Phase of a Vesicovaginal Fistula between the use of Freeze-Dried Amnion and Primary Suturing in Rabbit

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Abstract | Background: One of the complications of gynecological and obstetric procedures is a vesicovaginal fistula. Surgery is the gold standard but the success rate has not yet reached 100%. The wound healing process needs to be supported by certain ingredients such as regenerative medicine. Amnion is easy to find and rich in growth substances. **Aim:** This study analyzed the comparison of tissue structure in the remodeling phase of the vesicovaginal fistula between the use of freeze-dried amnion and primary suturing on day 21. **Materials and Methods:** This experimental study used New Zealand rabbits which were female and weighed 3-4.5 kg. The first stage of the study created a model of a vesicovaginal fistula. The second stage of the study closed the vesicovaginal fistula which was successfully created in the first stage. The study used a post-test-only control group design. The histological examination included fibroplasia, angiogenesis, re-epithelialization, and collagen deposition on day 21. Data were analyzed descriptively and statistically. **Results:** The mean scores of angiogenesis, fibroplasia, re-epithelialization, and collagen deposition in a New Zealand rabbit model of vesicovaginal fistula sutured with human amniotic stem cell seeding were higher than rabbits without amniotic stem cell seeding, the stem cell injection group, and the control group. There was a significant difference between groups only in angiogenesis at day 21. **Conclusion:** Freeze-dried amnion plays a role in the wound healing process, especially in angiogenesis in the vesicovaginal fistula repair model. The use of freeze-dried amnion can be applied to assist the wound healing process in patients but still needs to be studied in humans.

Keywords | Freeze-dried amnion, Wound healing, Vesicovaginal fistula, Histology, New Zealand rabbit model, Angiogenesis and obstetric disturbances

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INTRODUCTION

Vesicovaginal fistula, one of the complications of gynecological and obstetric procedures, is an abnormal opening between the bladder and vagina. These cases are still common in developing countries, with an estimate reaching at least three million women. Medical records in Africa show that 30,000-130,000 cases of vesicovaginal

fistula occur each year (Stamatakos et al., 2014). The results of a study in Uganda found that women suffred frm symptoms of vesicovaginal fistula for an average of 4.97 years with an average ICIQ_severity score of 7.21 (McCurdie et al., 2018). The mental health of the patient can be compromised due to the appearance of a vesicovaginal fistula causing significant morbidity. This is due to continuous leakage of urine (Colenbrande et al., 2021).

Although surgery is the gold standard, recurrence of vesicovaginal fistulas can occur. The treatment was not satisfactory (Ezeonu et al., 2017). The success rate of vesicovaginal fistula repair at the first attempt is about 89% (Warner et al., 2020). In some cases, such as induction radiotherapy, the results of surgical reconstruction are more than 90% and less than optimal (El-Azab et al., 2019). The recurrence of vesicovaginal fistula in Serbia showed there were 15 recurrent fistula formations including twelve after the first operation and three after the second operation (Hadzi-Djokic et al., 2009). In another study, 68.9% of patients had a primary fistula and 31.1% of patients had a recurrent fistula (Kumar et al., 2019). The development of a vesicovaginal fistula interferes with the hysterectomy process (Bodner-Adler et al., 2017).

Postoperative healing is a challenge in the healing process of vesicovaginal fistula. The success of the surgery is determined by the first attempt (Stamatakos et al., 2014). The process of wound healing, infection, and the immunological system are important points that determine the success of treatment (Lindberg et al., 2015). The wound healing process involves a complex and dynamic process, especially wound healing in the bladder area. This area will take a longer time to replace damaged tissue (Ninan et al., 2015). The remodeling phase is one of the phases in the complexity of wound healing. This phase lasts a year or more and begins two to three weeks after the onset of the lesion (Gonzalez et al., 2016).

Regenerative medicine is currently being developed, especially the utilization of stem cells from several parts of the human body. Amnion is a material that is easy to find and rich in growth substances for tissue repair. Amnion can be new potential material in supporting treatment, especially in pelvic floor disorders (Qiu et al., 2020). In previous studies, amnion has been used for wound healing through epithelial formation and protection from infection (Roubelakis et al., 2012). Dry amnion is useful in supporting ophthalmic surgery and has been shown to have logistical advantages over frozen amnion (Allen et al., 2013). Freeze-dried amniotic membrane has a better effect than cryopreserved amniotic membrane in the transplantation layer for treating corneal ulcers (Memmi et al., 2022). A comparison of tissue structure in the remodeling phase of the vesicovaginal fistula between the use of freeze-dried amnion and primary suturing on day 21 was analyzed in this study.

MATERIALS AND METHODS

Ethical approval

This experimental research on animals has received an ethics certificate No. 2.KE.016.01.2019 from the Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia.

Study period and location

This research was conducted at the Center for Stem Cell Research and Development, Universitas Airlangga for 6 months in 2019.

Dried amnion production

Cell study center Dr. Soetomo Academic Hospital provides Freeze Dried Amnion obtained from processed human amnion. Freeze-dried amnion is then stored in a freezer at a temperature of minus 4C. After drying, the amnion is then cut into pieces. The size used is 1 x 1 cm. Alpha-MEM was applied to the stem cell deposits and then resuspended. The resulting suspension was then added 1 ml portion from each well (2,000,000 stem cells/1 ml, according to the dose for rabbits) containing an amniotic membrane scaffold. As a result, 12 well plates were found which were incubated for 7 days. Two million doses of stem cells per ml for rabbits were carried out based on a study by Fuentes-Julián et al. (2015) which also involved rabbits (Fuentes-Julián et al., 2015).

Experimental animal preparation

The rabbit in this study was used as a model to be used as a human because the rabbit has some similarities with humans. In addition, rabbit wounds are easier to suture than mouse wounds. All New Zealand rabbits received the same food and drink during the week of adaptation and treatment after receiving treatment. The food given is pellets in the morning and evening with as much as 100g/kg BW. New Zealand female rabbits weight 3-4.5 kg were the sample of the study. Inclusion criteria were New Zealand female rabbits, a health condition characterized by soft fur, shining eyes, not weak, no scars, and a weight of 3-4,5 kg. Exclusion criteria were the rabbits had been tried as other animal research. Rabbits that were sick or died after receiving treatment and did not form vesicovaginal fistula were excluded from the sample. Table 1 describe the standardization of modeling of vesicovaginal fistula.

The production of fistula models

The fistula model was made by anesthetizing the New Zealand Rabbit by injecting ketamine 25-40 mg/kg BW and Azepromazine 0.25-1.0 mg/KgBW intramuscularly.

The prophylactic antibiotic enrofloxacin 10 mg/kg BW was also given intramuscularly. An incision was made in the anterior wall of the rabbit bladder for access after laparotomy and bladder identification. After treatment, the bladder wall was sutured with 4-0 screen sutures with simple interrupted sutures.

The rabbits were divided into 4 groups with each containing 12 rabbits. The control group was rabbits given layer-by-layer suturing treatment according to the main treatment of vesicovaginal fistula. The treatment group consisted of three groups. Treatment group 1 was rabbits given layer-by-layer suturing and the layer between the bladder and vagina was given amnion. Treatment group 2 was rabbits given layer-by-layer suturing and the layer between the bladder and vagina were given amnion that had been seeded with stem cells. Treatment group 3 was rabbits were given layer-by-layer suturing and injected with stem cells. Artificial fistula suturing as a treatment using 4-0 safyl threads. The stem cells used were amniotic stem cells $2x10^6$ in treatment group 2 and amniotic stem cells $1x10^6$ in treatment group 3.

Table 1: Standardization of modeling of vesicovaginal fistula

Surgery 1
Abdominal incision 5 cm
5 mm vaginal incision
5 mm posterior bladder incision
Insertion of NGT 16 Fr, connecting the vagina and vesica
Vaginal and vesical suturing, simple interrupted suture, watertight
Leak test
The abdomen is closed layer by layer
The rest of the NGT is cut, fixation on the labia
3 weeks treatment
Surgery 2
Repeat laparotomy
Anterior blade in 1cm incision
Repair fistula formed
Anterior vesica is sewn 2 layers
Leak test
Clinical evaluation of diet, defecation, urination, and signs of a sick rabbit

Histological examination

New Zealand rabbits were evaluated 21 days post-treatment for histological characterization examination. The histological staining process was carried out through five key stages, namely fixation, processing, embedding, sectioning, and staining. Over time there has been an increase in histopathology and histotechnology in the stains used. Many staining procedures are still in use today, and many others have been replaced by other advanced immunostaining, molecular, non-cultural and staining techniques. Some staining methods have been abandoned because the chemicals required have been shown to be medically toxic. Case studies show that in modern histology a combination of different staining techniques is used to increase the effectiveness of the staining process. Hematoxylin is a basic dye that is commonly used in this process and stains the nuclei giving it a bluish color while eosin (another stain dye used in histology) stains the cell's nucleus giving it a pinkish stain (Alturkistani et al., 2015).

Histological examination in this study was using Hematoxylin-Eosin examination. Histopathological examination was performed to assess the score of fibroplasia, angiogenesis, re-epithelialization, and collagen deposition. The examination was carried out through semi-quantitative counting viewed under a microscope with a magnification of 100 X. The examination was carried out with the Abramov score. The score was calculated as 0-3 degrees. The degree of fibroplasia was seen from the shape and arrangement of fibroblasts. Mature fibroblasts are thin and usually tightly packed and parallel. Immature fibroblasts are star-shaped and irregular. Re-epithelialization was assessed based on the condition of the epithelial tissue, epithelial migration, mitotic activity in the wound area, and its thickness. Angiogenesis was assessed by the number of blood vessels formed in each visual field. Collagen deposition was assessed using the Abramov score. The score was calculated with degrees 0-3. Collagen deposition was seen based on the density of collagen formed.

Postmortem changes detected during running this experiment using several indicators. Indicators of the well-being of the rabbit's physical condition were monitored during post-mortem examination. This examination is done to assess the presence of cuts, bruises, scratches, abscesses, dermatitis and signs of infection (Valcova et al., 2021). In this study, the mortality rate was found to be one in forty-eight rabbits as a result of the creation of a vesicovaginal fistula. This rabbit was included in the control group. This is because the rabbits had a deteriorating condition during the study.

Statistical analysis

The Kolmogorov-Smirnov test or the Shapiro-Wilk test were used to test for normality. A one-way ANOVA test was used to compare the mean of the dependent variable between the 3 groups if the data were normally distributed ($p>0.05$). After the ANOVA test, data were further analyzed using the post hoc Least Significant Difference (LSD) test. Kruskal-Wallis test was used if the data were not normally distributed ($p<0.05$). After the Kruskal-Wallis test,

Table 2: Statistical analysis of angiogenesis scores, fibroblast maturation, re-epithelialization, and collagen deposition day-21

Group	Frequency (n)	21st-day test results	Statistical test (Kruskal -Wallis)
Re-epithelialization			
Control	6	2,00 (0-3)	p= 0.055
Treatment 1	6	2,00 (1-3)	
Treatment 2	6	3,00 (3-3)	
Treatment 3	6	3,00 (1-3)	
Angiogenesis			
Control	6	1,00 (1-1)	p= 0.018
Treatment 1	6	1,00 (1-2)	
Treatment 2	6	2,00 (1-2)	
Treatment 3	6	1,00(1-2)	
Fibroblast maturation			
Control	6	1,50 (0-2)	p= 0.051
Treatment 1	6	2,00 (1-3)	
Treatment 2	6	3,00 (2-3)	
Treatment 3	6	2,50 (1-3)	
Collagen maturation			
Control	6	2,00 (1-2)	p= 0.091
Treatment 1	6	2,50 (1-3)	
Treatment 2	6	3,00 (2-3)	
Treatment 3	6	2,50 (2-3)	

data were further analyzed using the Mann-Whitney post hoc test. Statistical calculations will be used with computer software.

RESULTS

The results of postmortem examination showed that during the study the rabbits were in good health except 1 rabbit from the control group which had to be replaced with another rabbit. Mean scores of angiogenesis, fibroplasia, re-epithelialization, and collagen deposition in New Zealand rabbit models of vesicovaginal fistula that were sutured with human amniotic stem cell seeding were higher than those without amniotic stem cell seeding, the stem cell injection group, and the control group. On day 21, re-epithelialization of the control group (primary suturing) was the lowest with a mean score of 1.67. Meanwhile, treatment 2 (dry amnion stem cell seeding) was the highest score with a score of 3. Angiogenesis in the control group (primary suturing) was the lowest with an average score of 1.00. While in treatment 2 (dry amnion stem cell seeding) was the highest score with a value of 1.83. On day 21, the fibroplasia of the control group (primary suturing) was the lowest with a mean score of 1.33. While in treatment 2 (dry amnion stem cell seeding) was the highest score with a value of 2.67. On day 21, collagen in the control group (primary suturing) was the lowest with a mean score of 1.67. While in treatment 2 (dry amnion stem cell seeding) was the highest score with a value of 2.67. Further analysis regarding the comparison of the average scores of angiogenesis, fibroplasia, re-epithelialization, and collagen deposition on day 21 between groups is shown in Table 2. Fibroplasia, re-epithelialization, and collagen deposition scores were not significantly different between groups. Significant differences were seen in the angiogenesis scores.

The number of fibroblast cells, the number of collagen cells and the results of re-epithelialization, showed better results in the treatment group group 2. These results are included in Figures 1 and Figure 2. Figure 1/KH21 shows severe congestion and hemorrhage with profuse fibroblasts, Figure 1/P3H21 shows cystic dilatation, epithelial cell necrosis and sloughing and Figure 1/P1H21 shows hyperactivation of goblet cells Observations were carried out qualitatively using scores from Greenhalgh (Table 1). The data were collected from 3 (three) different visual fields with 100x (reepithelialization) and 400x (other) magnification. All of these examinations used an ordinary Nikon C-H2L Eclipse Ci light microscope which was equipped with a 300 megapixel DS Fi2 digital camera and image processing software Nikkon Image System.

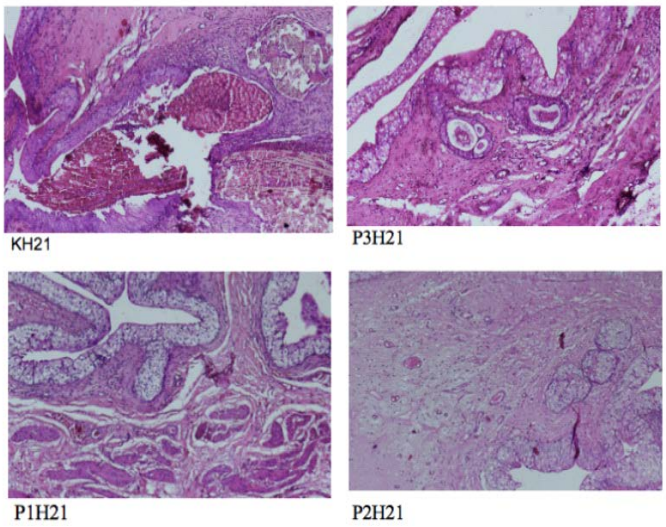

Figure 1: Histopathological examination of reepithelialization

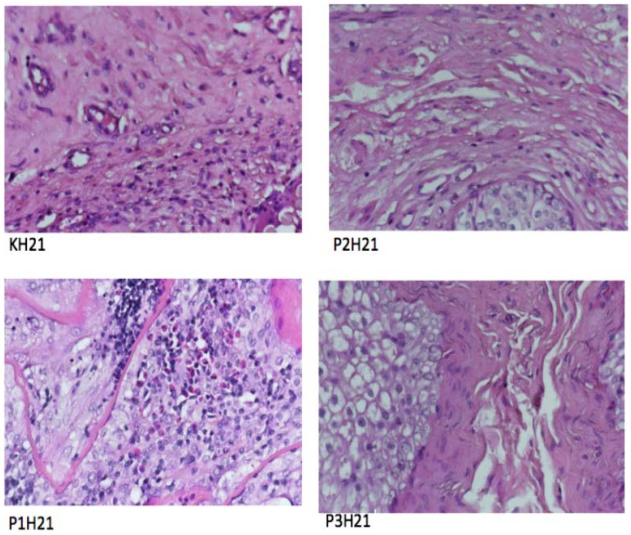

Figure 2: Histopathological examination of angiogenesis, fibroplasia and collagen deposition

DISCUSSION

Freeze-dried amnion plays a role in the wound healing process, especially in angiogenesis in the vesicovaginal fistula repair model. Tissue structure on day 21 in primary suturing plus freeze-dried amnion is more than in primary suturing. This is a new finding because previous studies examined the remodeling phase at 7 days or 14 days. The remodeling phase takes a long time to process. These phase in the normal wound healing process takes about one to two years, or even longer (Ayavoo et al., 2021). This is because the healing process is modulated by exogenous and endogenous factors. Systemic disorders, hypertrophic scars, and keloids as well as external factors such as corticotherapy and smoking can interfere with the initial wound closure process (Gonzalez et al., 2016). The right decisions, the expertise of health professionals, methods of repair, appropriate preoperative evaluation, and careful postoperative management are the determinants of success. Freeze-dried human amnion can can help in every wound healing process (Kurniawati et al., 2022).

Angiogenesis

Examination of re-epithelialization, angiogenesis, fibroplasia, and collagen deposition showed histological improvement in each examination, although there was no significant difference only at day 21 of angiogenesis. Angiogenesis is the growth of blood vessels from existing blood vessels (Adair et al., 2010). Wound clots containing large amounts of fibrin/fibronectin are invaded by angiogenic capillary buds during wound healing. Microvascular tissue forms throughout the granulation tissue within a few days (Tonnesen et al., 2000). Endothelial survival, proliferation, and migration are the main factors for angiogenic stimulation through increased soluble factor mediation (Demidova-Rice et al., 2012). The process of angiogenesis occurs because it begins with the initiation of Vascular Endothelial Growth Factors (VEGFs) and Placental Growth Factors (PlGFs) (Naicker et al., 2019). Pathology of female reproductive organs also affects the occurrence of angiogenesis disorders (Reynolds et al., 2002). The process of angiogenesis involves complex factors and is related to other growth factors.

Fibroplasia

The connective tissue cells that contribute to repairing injured tissue are fibroblasts. Fibroblasts contribute to collagen deposition. There was no significant difference in fibroplasia scores. These results indicate that the use of dry amnion does not assist in the fibroplasia process although there is visible improvement in wound healing tissue. Fibroblasts are critical in supporting normal wound healing, involved in key processes such as breaking down the fibrin clot, creating new extra cellular matrix (ECM) and collagen structures to support the other cells associated with effective wound healing, as well as contracting the wound (Bainbridge, 2013). Healing problems such as fibrosis and delayed healing may occur if fibroblast dysfunction is present (Cialdai et al., 2022).

Re-epithelialization

There was no significant difference in re-epithelialization scores. These results indicate that the use of dry amnion does not assist in the re-epithelialization process. Re-epithelialization is a process that involves the migration and proliferation of keratinocytes which helps the process of tissue recovery to be intact from an open wound due to injury (Li et al., 2007). Chronic wounds and ulcers are a form of complication due to the failure of the re-epithelialization process.

Collagen deposition

There were no significant differences in scores of collagen deposition. Another study found that there was some collagen deposition on day 7 post-injury found in the exosomes group. Some irregular collagen and fibroblast proliferation appeared after day 14 in wounds that are not treated with stem cells (El-Tookhy et al., 2017). Collagen forms a major part of the extracellular matrix along with glycosaminoglycans, proteoglycans, laminins, fibronectin, elastin, and cellular components. Collagen is the most abundant triple helix protein molecule in animal tissues (Ricard-Blum, 2011).

Stem cells play an active role in inducing collagen matrix and transcription of collagen I and collagen III. Collagen fibers were denser and thicker at day 21, indicating the maturation phase of wound healing (Borena et al., 2010). Improvement was started on the third day of wound healing in rabbit skin that had burns after being given stem cells. This indicates an increase in vascularity and collagen deposition (Bliley et al., 2016).

LIMITATIONS AND RECOMMENDATION

This study was conducted on test animals and has not been tested on humans so the effect of vesicovaginal fistula in humans is unknown. Further research needs to examine other parameters related to the wound healing phase, human studies, and the standardized mechanism of making freeze-dried amnion.

CONCLUSION

There was an increase in the results of histological examination, namely re-epithelialization, angiogenesis, fibroblast maturation, and collagen deposition, but the significant difference was only found in angiogenesis at day 21. The use of freeze-dried amnion can be applied in urogynecology and pelvic floor disorders to assist the wound healing process in patients but still needs to be studied in humans.

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DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary material.

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CONFLICT OF INTEREST

There is no conflict of interest.

AUTHOR CONTRIBUTION

EMK: concept and design, drafting manuscript, BS and FAR: supervision, WDJ : Critical revision of manuscript, BIS : final approval and drafting manuscript THS and GH : admin, technical or material support, HAP: Funding and manuscript review.

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