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Effect of Freeze-dried Amnion with Human Amniotic Stem Cells Seeding on the Expression of FGF-2 and the Number of Fibroblasts in Vesicovaginal Fistula: An Animal Study

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ABSTRACT

Surgery is the standard therapy for vesicovaginal fistula. The surgical success rate in these cases ranges from 70-100%, and the risk of recurrence is high. The human amniotic membrane is especially being developed for tissue engineering in urogynecology. The study aimed to determine the effect of freeze-dried amnion with human amniotic stem cells seeding on the expression of FGF-2 and the number of fibroblasts in vesicovaginal fistula in New Zealand rabbits. We conducted experimental research with a post-test-only control group design. The female rabbits were divided into three groups, each consisting of 12 rabbits. The specimens taken were subjected to immunohistochemical staining to determine the expression of FGF-2 and hematoxylin-eosin staining to determine the number of fibroblast cells. We analyzed data statistically. The expression of FGF-2 in primary suturing and freeze-dried amnion with human amniotic stem cell seeding was higher than in the other groups ($P<0.05$). We found the highest fibroblast cells in the primary suturing and freeze-dried amnion with human amniotic stem cell seeding ($P<0.05$). The use of freeze-dried amnion with the seeding of human amniotic stem cells affects postoperative wound healing seen from the expression of FGF-2 and the number of fibroblasts. This study can be used as a recommendation for clinical use but needs to be clarified by conducting human studies.

Key words: Amnion, FGF-2, Fibroblast, Vesicovaginal fistula, Rabbit.

INTRODUCTION

A vesicovaginal fistula is an abnormal communication between the bladder and the vagina that causes continuous urination (Rajaian et al. 2019). Vesicovaginal fistula cases are still common in developing countries, often due to obstetric complications, such as obstructed labor (Stamatakos 2014). Vesicovaginal fistulas occur due to iatrogenic conditions during hysterectomy or after obstetric trauma (Agil and Kurniawana 2022). Vesicovaginal fistulas are rare in developed countries (El-Azab et al. 2019). The incidence of vesicovaginal fistulas in developing countries has an estimated incidence of one to two per 1000 deliveries and an annual incidence of 50,000-100,000 cases, while untreated fistula cases are 500,000-2,000,000 (Hilton 2003; Abrams et al. 2010).

Vesicovaginal fistulas considerably impact sufferers, especially social relationships such as divorce, disturbed intimate relationships, and depression (Adler et al. 2013). Vesicovaginal fistula is one of the most distressing conditions for women. This case causes a lot of social, emotional, and psychological stress and strain on the patient. Stress incontinence is most likely to occur in obstetric fistulas where the injury disrupts the sphincter mechanism (Malik et al. 2018).

Surgical treatment is the primary method of vesicovaginal fistula repair (El-Azab et al. 2019). Failure of repair and fistula recurrence can occur in 30% of cases (Malik et al. 2018). The treatment success rate is 70-100% in cases of non-irradiated vesicovaginal fistula. Fistula recurrence can occur within three months after the first surgery (Yuh and Rothschild 2016; Adler et al. 2017). Surgical closure is the gold standard of treatment, although successful closure only occurs in about 85% of patients after the first repair. Recurrence remains a troublesome complication for the patient and the surgeon (Streit-Ciećkiewicz et al. 2019). Achieving a 100% closure

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rate and addressing continence issues in managing vesicovaginal fistulas remains challenging (Breen and Ingber 2019).

Wound healing is one of the most common health problems because treatment costs have increased over the last decade. In addition, wound healing is a complex process. Wound healing involves a process of hemostasis (Guo et al. 2021). Wound healing goes through 4 phases, including hemostasis, inflammation, proliferation, and remodeling (Enoch and Leaper 2008). In the proliferative phase, granulation tissue is formed consisting of new capillaries, fibroblasts, and macrophages (Prasetyono 2009). The development of materials that support wound healing can support the success of therapy (Guo et al. 2021).

Activation and proliferation of fibroblasts are stimulated by Fibroblast Growth Factor (FGF), Platelet-Derived Growth Factor (PDGF) and Epidermal Growth Factor (EGF) (Chatterjee et al. 2019). FGF-2 has been shown to regulate many cellular functions, including cell proliferation, migration, and differentiation, as well as angiogenesis in various tissues, including skin, blood vessels, muscles, adipose, tendons/ligaments, cartilage, bones, teeth, and nerve (Benington et al. 2020). FGF-2 is an adipokine that can impair adipocyte inflammatory response (Zhuge et al. 2020). FGF-2 also accelerates wound closure by activating vascular endothelial cells and fibroblasts (Koike et al. 2020).

Tissue engineering is the application of engineering and science to restore tissue function. It has an important component, biomaterials or scaffolds, that function as supports and sites for cell growth (Langer et al. 2008). The human amniotic membrane is one of the leading candidate materials used in tissue engineering, both as a biological dressing and/or scaffold and as a source of mesenchymal stem cells because it is easy to obtain, non-invasive, easy to process, and transport. The amniotic membrane was chosen as a scaffold because it contains growth factors, has good biocompatibility, antimicrobial effect, low immunogenicity, and has an extracellular matrix that causes better cell attachment. Freeze-dried amnion is a scaffold derived from the human amniotic membrane that has undergone a series of processes so that the growth factor content remains (Niknejad et al. 2008). Freeze-dried amnion has the advantage that it can be stored and is more stable in various storage conditions without losing growth factor content so that it can be used as a scaffold in tissue reconstruction (Ihsan and Prijanto 2009). Wound dressings are based on decellularized biomaterials, which are the focus of regenerative medicine as natural ingredients. In particular, human amniotic are easily accessible and without ethical restrictions (Xiao et al. 2021).

The amniotic membrane is also used as a source of mesenchymal stem cells. Mesenchymal stem cells have an important role in assisting the wound healing process because they can secrete various mediators in tissue repair, including growth factors, cytokines, and chemokines, especially FGF-2, PDGF, EGF, Vascular Endothelial Growth Factor (VEGF), Keratinocyte Growth Factor (KGF), and Transforming Growth Factor-β (TGF-β). Mesenchymal stem cells also have paracrine signals to which several cell types can respond. One of them is fibroblasts, which affect the process of cell migration and proliferation in vitro. Mesenchymal stem cells act as chemo-attractants for macrophages, fibroblasts, endothelial cells, and keratinocytes (Maxson et al. 2012).

Tissue engineering in urogynaecology is a potential topic and is currently developing. This study can provide evidence based on the role of freeze-dried amnion with human amniotic stem cells seeding when viewed from the expression of FGF-2 and the number of fibroblasts in vesicovaginal fistulas. These results become the basis for the development of further research that needs to be done on humans. Research on the use of stem cells derived from the human amniotic membrane in cases of vesicovaginal fistula has not been widely carried out. This study aims to determine the effect of freeze-dried amnion with human amniotic stem cells seeding on FGF-2 expression and the number of fibroblasts in vesicovaginal fistula in New Zealand rabbits.

MATERIALS AND METHODS

Ethical approval

This research carried out an ethical test at the Research Ethics Commission of the Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia.

Research design

Experimental laboratory research was conducted with female New Zealand rabbits as experimental animals. The research design used was a posttest-only control group design. This research was carried out in the Pathology Laboratory of the Faculty of Veterinary Medicine, Universitas Airlangga, and the Stem Cell Laboratory at the Institute of Tropical Disease, Universitas Airlangga. Samples were divided into 3 groups with 1 group containing 12 rabbits. The control group (K) only underwent primary suturing of the fistula. Treatment group 1 (P1) underwent primary suturing and was given freeze-dried amnion without amniotic stem cell seeding. Treatment group 2 (P2) underwent primary suturing and was given freeze-dried amnion with amniotic stem cell seeding.

Inclusion and exclusion criteria

The sample of this study was New Zealand female rabbits weighing 3-4kg, adapted for one week. The number of samples in this study was 12 samples from each group. The inclusion criteria were healthy conditioned New Zealand female rabbits characterized by soft fur, shining eyes, no limp, no scars, and rabbits with a vesicovaginal fistula after modeling. Rabbits used as experimental animals in other studies are not used as samples. Rabbits that were sick or died after receiving treatment were excluded from the list of research samples.

Research flow

Vesicovaginal fistula models in all groups were made by making a 5mm defect through laparotomy and then suturing the vaginal and bladder mucosa with 4-0 safyl sutures. The defect formed was maintained using a 16Fr Naso Gastric Tube (NGT) for three weeks. The control group (K) only underwent primary suturing of the fistula. Treatment group 1 (P1) underwent primary suturing and

was given freeze-dried amnion without amniotic stem cell seeding. Treatment group 2 (P2) underwent primary suturing and was given freeze-dried amnion with amniotic stem cell seeding. Surgery was performed on the 7th day after treatment to obtain the study sample. The primary suturing was the layer-by-layer suturing of the vesicovaginal fistula using a 4-0 sapphire suture and a simple interrupted suture technique. The suturing of the vesicovaginal fistula with the addition of freeze-dried amnion was performed by layer-by-layer suturing of the vesicovaginal fistula using a 4-0 sapphire suture and a simple interrupted suture technique by placing a 1x1cm freeze-dried amnion between the vaginal wall and bladder. The suturing of the vesicovaginal fistula with the addition of freeze-dried amnion and human amniotic stem cell seeding was performed layer-by-layer. Then, the vesicovaginal fistula was sutured using a 4-0 sapphire suture and a simple interrupted suture technique by placing a 1x1cm freeze-dried amnion implanted and then sown with amniotic stem cells ($1x10^6$) between the walls of the vagina and bladder. After 7 days, FGF-2 and fibroblast cell count were examined.

The expression of immunoreactive cells expressing FGF-2 was indicated by the presence of a brown color representing the binding of antigens and antibodies. The procedure was carried out according to the modified Remmele method (Novak et al. 2007). The number of fibroblasts was counted in the granulation tissue. After hematoxylin-eosin (HE) staining, the average number of fibroblasts was performed by looking under a microscope with a magnification of 100x.

Statistical Analysis

Data were analyzed descriptively and statistically. The normality test was carried out using the Shapiro-Wilk test. Normally distributed data ($P>0.05$) were tested with one-way ANOVA to see the homogeneity of the variants of the three groups. If the homogeneity of the variance was not different, it was to be continued with the post hoc Least significant difference (LSD) test. Data that are not normally distributed ($P<0.05$) were tested with Kruskal-Wallis then, followed by the Mann-Whitney post hoc test. Calculation statistics using the SPSS 21 tool.

RESULTS

The New Zealand rabbit used in this study was a female rabbit with a weight ranging from 3-4kg according to the inclusion criteria. The average weight of rabbits in the control group was 3.37kg, treatment group 1 was 3.37kg, and treatment group 2 was 3.47kg. The results of the Shapiro-Wilk test showed no difference in weight variance between groups in rabbits.

The strongest FGF-2 expression was found in treatment group 2, which appeared in medium-strong brown color intensity with many positive immunoreactive cells. In contrast, the lowest was found in the control group, which showed no-low color intensity with few cells with positive immunoreactivity. The results of the data normality test showed that the FGF-2 expressions of the three groups were normally distributed, with $P>0.05$. However, the variance homogeneity test found different variants between groups, indicated by $P<0.05$. Kruskal-Wallis, followed by the post hoc Mann-Whitney test, was used to determine the comparison of FGF-2 expression between groups.

The number of fibroblast cells was highest in treatment group 2 and the least in the control group. The homogeneity of variance test results showed different variants with $P=0.055$, so it was continued with the Kruskal-Wallis and post hoc Mann-Whitney statistical tests to determine the differences between groups. Statistical analysis results between the control and treatment groups 1 showed a significant difference with $P=0.01$. The same results also showed the comparison of the number of fibroblasts between the control and treatment group 2 ($P<0.001$) and between treatment group 1 and treatment 2 ($P=0.02$). The most significant results were obtained between the control and treatment group 2, with the most significant difference in the mean value and the smallest p-value.

DISCUSSION

Vesicovaginal fistula is a medical problem in the field of urogynecology. An abnormal connection between the bladder and the vagina causes the involuntary leakage of urine (Santoso 2011). Surgery is the standard therapy for vesicovaginal fistulas with a high success rate, but postoperative fistula recurrence is quite challenging. Recurrence can occur about three months postoperatively and the number, size, and tissue around the fistula could be etiology (Zhou et al. 2017). Tissue engineering has great potential in urogynecology (Wu et al. 2020).

The use of freeze-dried amnion with human amniotic stem cell seeding affects postoperative wound healing seen from the expression of FGF-2 and the number of fibroblasts. Human freeze-dried amnion plays a role in the wound-healing process in vesicovaginal fistula repair models (Kurniawati et al. 2022). The human amnion has growth properties, cytokines, and cells similar to stem cells. This important source of scaffolding material has been studied and used extensively in various areas of tissue repair, namely corneal repair, chronic wound care, genital reconstruction, tendon repair, microvascular reconstruction, nerve repair, and intraoral reconstruction (Fénelon et al. 2021).

The results showed significant differences in the expression of FGF-2 in all groups. The treatment group that underwent primary suturing and freeze-dried amnion with stem cell seeding had a higher FGF-2 expression than the group that only underwent primary suturing. This study follows a study by Padeta et al. (2017), which showed that the FGF-2 expression in the burn model of mice treated with mesenchymal stem cells was higher than that of mice treated with mesenchymal stem cells burns (Padeta et al. 2017). Another study by Perdanakusuma and Febrini (2013) also stated that the FGF-2 receptor was more commonly found in the skin of injured rabbits and given mesenchymal stem cell therapy compared to the group that only used standard therapy (Perdanakusuma and Febrini 2013). In accelerating growth, the required FGF receptor is a receptor tyrosine kinase. These receptors are involved in several biological processes, such as the regulation of tissue development and repair (Katoh 2019). Mesenchymal stem cells are

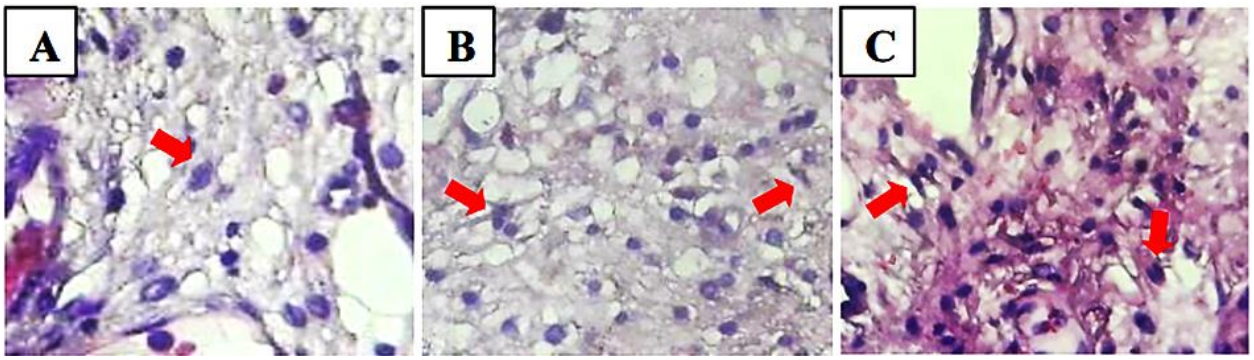

Fig. 2: Fibroblast cells with hematoxylin-eosin staining. (A) control group; (B) treatment 1; and (C) treatment 2. Data for each sample was taken using a 400x magnification microscope.

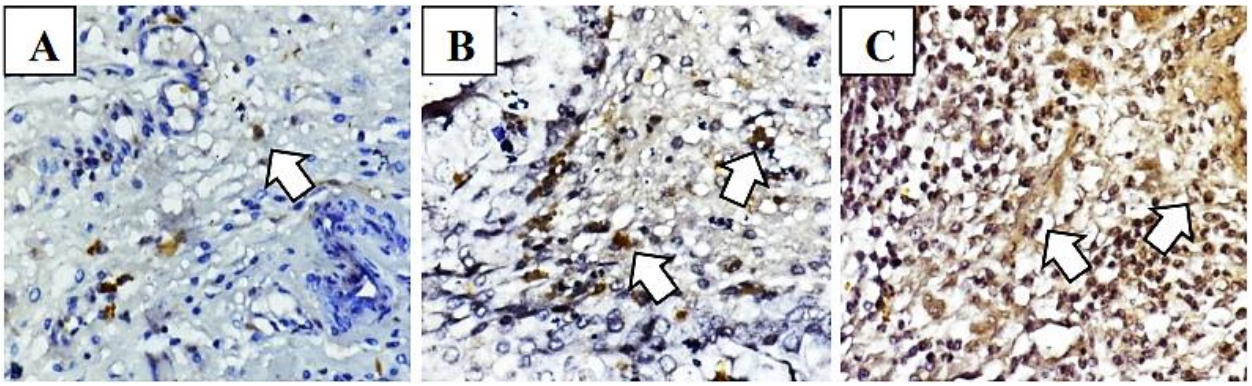

Fig. 1: Differences in FGF-2 expression in the vaginal lamina propria by immunohistochemical staining indicated by the presence of chromogen brown color (arrow). Data for each sample was taken using a 400x magnification microscope.

Table 1: Results of immunohistochemical statistical analysis of FGF-2 expression and number of fibroblast cells

Group	Median	Mean	Minimum	Maximum	P-value
Immunohistochemistry of FGF-2 expression					
Control	1.20	1.23	0.2	2.3	P<0.001
Experiment 1[b]	4.05	4.02	1.8	8.5	
Experiment 2[c]	6.6	6.12	3.6	8.2	
Fibroblast cell count					
Control	11.0	12.25	9	19	P<0.001
Experiment 1[b]	16.5	16.67	10	25	
Experiment 2[c]	24.5	22.42	12	29	

The Kruskal-Wallis test was applied. [a]Primary suturing; [b]Primary suturing + freeze-dried amnion; primary suturing + freeze-dried amnion + stem cell seeding. Statistical analysis results between the control group and treatment group 1 showed a significant difference with P<0.001.

Table 2: Results of comparative analysis of FGF-2 expression and the number of fibroblasts between groups

Group	Mean	P-value
FGF-2 expression		
Treatment 1 vs. control	2.79	P<0.001
Treatment 2 vs. control	4.89	P<0.001
Treatment 2 vs. treatment 1	2.1	P=0.028
Fibroblast cell count		
Treatment 1 vs. control	4.42	P<0.010
Treatment 2 vs. control	10.17	P<0.001
Treatment 2 vs. treatment 1	5.75	P=0.020

Uji Mann-Whitney test applied. The examination results of the number of fibroblast cells were carried out quantitatively in each sample observed in five fields.

important in wound-healing, especially in the proliferative phase. These stem cells express several growth factors, one of which is FGF-2 which functions to induce the granulation process (Maxson et al. 2012). Fibroblast growth factor-2 will bind to its receptor resulting in the dimerization of the receptor. The interaction between FGF-2 and its receptor will activate the phosphatidylinositol-3 kinase and mitogen-activated protein kinase pathways. This activation increases cell migration and proliferation (Demidova-Rice et al. 2012).

FGF-2 acts as an early mediator released in the wound area, so FGF-2 plays an important role in early wound healing. Akita et al. (2008) showed that second-degree burns that were given FGF-2 experienced faster-wound healing than the control group. This mechanism is carried out by providing better granulation tissue regeneration and forming new blood vessels. (Akita et al. 2008). This growth factor is produced by inflammatory cells, endothelial cells, fibroblasts, and keratinocytes. The mechanism of FGF-2 in the wound healing process has been known both in vitro and in vivo, namely through its mechanism in activating local macrophages, increasing the production of extracellular matrix components, fibroblast proliferation, proliferation and migration of

endothelial cells for angiogenesis and re-epithelialization (Cornick et al. 2014). The study by Tanaka et al. (1996) showed a decreased amount of FGF-2 in a diabetic rat model. This decrease in FGF-2 will cause wound healing to be hampered and continue to become chronic wounds (Tanaka et al. 1996). Leila et al. (2017), who applied the amniotic membrane to patients with perianal fistulas who underwent fistulotomy, showed better-wound healing than patients with standard treatment (Leila et al. 2017). ElHeneidy et al. (2016) also obtained the similar results, which applied the amniotic membrane to chronic ulcers on the feet, namely faster-wound healing time with more healthy granulation tissue in patients treated with human amniotic membranes (ElHeneidy et al. 2016). Research by Kakabadze et al. (2019) showed that wounds in rats treated with mesenchymal stem cells prepared in freeze-dried amnion had a healing time of 4 times faster than freeze-dried amnion without stem cells (Kakabadze et al. 2019).

Freeze-dried amnion is a human amniotic membrane that has undergone a drying and sterilization process using -rays so that it can be stored at room temperature and last longer but still contains growth factors that play an important role in the wound healing process (Chopra and Thomas 2013). Forming the amniotic membrane into a freeze-dried amnion requires a series of processes, from freezing, freeze-drying, and radiation for sterilization. Research by Ihsan and Prijanto (2009) found a lower amount of growth factor in freeze-dried amnion when compared to fresh amnion, which could be caused by a series of processes in its manufacture (Ihsan and Prijanto 2009). Another study stated that freezing or cryopreservation at -80^{o}C and sterilization using - radiation could reduce the amount of FGF-2 in freeze-dried amnion (Pasaribu 2012; Paolin et al. 2016).

The examination of the number of fibroblasts in this study showed that the number of fibroblasts in the group that underwent primary suturing and the administration of freeze-dried amnion without stem cell seeding was significantly higher than the group that only underwent primary suturing. These results are consistent with Rinastiti (2003) research, which showed that on the seventh day, the number of fibroblast cells in the freeze-dried amnion group in injured gums was significantly higher than in the control group who were not given freeze-dried amnion (Rinastiti 2003). A significantly higher number of fibroblast cells was also demonstrated in a rat study of a colon anastomosis model wrapped with freeze-dried amnion compared to a control group that only had primary suturing (Rizali 2018).

Fibroblasts are one of the factors that influence the proliferation process, which helps tissue regeneration by producing collagen and extracellular matrix. Fibroblasts are in the wound area and proliferate so that the granulation tissue is rich in fibroblasts. The process of migration and proliferation of fibroblasts occurs due to the presence of growth factors. The amniotic membrane contains many growth factors that accelerate the migration and proliferation process of fibroblasts. The use of amnion will encourage the wound healing process to take place faster and better (Rinastiti 2003). This study also showed that groups treated with primary suturing and the administration of freeze-dried amnion with stem cell seeding had significantly more fibroblast cells than those that underwent primary suturing with freeze-dried amnion without stem cell seeding and the group that was sutured primarily only. The following research by Mohamed et al. (2019) showed that stem cells seeded on the scaffold had better wound healing, namely that the resulting granulation tissue contained more fibroblasts compared to the wound group that was only given scaffolding and the control group, which was not given anything (Mohamed et al. 2019).

Mesenchymal stem cells secrete a variety of mediators that play a role in the wound-healing process, including cytokines, chemokines, and growth factors. Mesenchymal stem cells also secrete mitogenic substances such as FGF-2 and TGF-β that cause the proliferation of fibroblasts, keratinocytes, and endothelial cells. Mesenchymal stem cells have paracrine signaling and the ability to differentiate. The ability of stem cells to differentiate contributes to tissue regeneration, while the paracrine signals possessed by stem cells regulate cell responses in the wound area. Both of these are mechanisms that mesenchymal stem cells can increase tissue repair and are one of the advantages of the role of mesenchymal stem cells in wound healing by reducing inflammatory reactions, inducing migration and proliferation of cells, including fibroblast cells, and stimulating angiogenesis (Maxson et al. 2012; Sanjari et al. 2015). Human freeze-dried amnion plays a role in the wound-healing process in vesicovaginal fistula repair models. It is hoped that this research will improve urogynecology services (Kurniawati et al. 2022).

The human amniotic membrane plays an important role in tissue engineering. The human amniotic membrane can be used as a biomaterial or scaffold and a source of stem cells. The amniotic membrane was chosen because it is easier to obtain and non-invasive by taking it from delivery; it is easy to process and transfer (Niknejad et al. 2008). Human amniotic epithelial cells have been tested in obstetrics and gynecology and achieved the expected results. This use can be a new disease treatment and deserves in-depth research and exploration (Xuan et al. 2021).

In this study, experimental animals in the form of a New Zealand female rabbit were used as a model for a vesicovaginal fistula. Rabbits were chosen because they are easier to care for, do not take up space, and have separate vesical and vaginal anatomy, so dissection is needed before vesicovaginal fistulas are made. The vesicovaginal fistula that had been created was maintained for 3 weeks using NG 16Fr, which was then treated according to the group. Rabbits are often included in biomedical research, particularly as a bioreactor for producing antibodies. The principle of using rabbits rather than other animals as an experimental model is that rabbits are the only animals used for translational research, which are difficult to replace with other species (Fan et al. 2018).

Conclusion

The use of freeze-dried amnion with the seeding of human amniotic stem cells affects postoperative wound healing seen from the expression of FGF-2 and the number of fibroblasts. This study can be used as a

recommendation for clinical use but needs to be done in human studies.

Limitation and recommendation

This study has several limitations that can be used as improvements in further research, including the absence of stem cell homing examinations in fistula vesicovaginal suturing and the absence of an objective examination of the condition of the wound after treatment so that the speed of wound healing cannot be assessed. Recommendations for further research are to examine the stem cell homing used, conduct an objective assessment after vesicovaginal fistula suturing and examine the different parameters to assess the effect of FGF-2 on the wound healing process, especially in the proliferative phase.

Author's Contribution

EMK and BS conceived of the presented idea. FAR developed the theory. BIS and WID verified the analytical methods. EMK, THS, GH and HP carried out the experiment. All authors discussed the results and contributed to the final manuscript.

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