Freeze-dried Amniotic Membrane (FD-AM) Application to Fibroblast Growth Factor Expression on Urethral Defect Reconstruction in New Zealand White Rabbit (*Oryctolagus cuniculus*)

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Abstract

Introduction: Urethral reconstruction remains a challenge for surgeons. Freeze-Dried Amniotic Membrane (FD-AM) has often been used as a scaffold for the substitution of damaged tissue. Fibroblast Growth Factor (FGF) assists collagen synthesis in the proliferation phase of wound healing phase. This study aims to determine the importance of the role of fibroblasts in the healing process of urethral defects closed by FD-AM patch.
Methods: This study is an experimental study. Total of 36 male rabbits (*Oryctolagus sp.*) underwent urethroplasty, of which 18 underwent urethral defect reconstruction with FD-AM patches and others underwent primary repair of urethral defects. After 28 days, the urethral wound was observed and the specimen was taken for histopathological examination to calculate the amount of FGF histochemically.
Result: There was no relationship between FGF and wound healing in both groups (Spearman test; p=0.670 treatment group; p=0.757 control group).
Conclusion: No significant relationship was found between increased FGF and wound healing in this study.
Key words: Urethral Defect,;FD-AM; FGF

1. Introduction

Urethral reconstruction is still a challenge for surgeons since ancient times where various reconstruction techniques are used with varying success rates and quite a lot of therapeutic failures. Various complications that often occur, such as hair growth, strictures, litho-genesis, and the formation of diverticula.[1] FD-AM is widely used as a scaffold for the substitution of damaged tissue. FD-AM has been used in ophthalmological surgery, as a wound dressing, vestibuloplasty, and even in neurosurgical procedures as a dura mater replacement in myelomeningocele surgery. The use of FD-AM as a scaffold to close urethral defects in experiment using rabbits has also been carried out.[2] In this study, it was said that FD-AM was proven to be able to grow and become tissue that covered the urethral defect. The growth of FD-AM into urethral tissue is much influenced by growth factors, local tissue conditions, surgical techniques, and thread material used. In the process of healing urethral wounds, the growth of FD-AM into urethral tissue is strongly influenced by interrelated factors, from inflammation to maturation. Two weeks after going through the inflammatory phase, a proliferation phase occurs, namely the start of myofibroblast proliferation, angiogenesis, and collagen production, especially collagen III. Collagen is the most abundant protein in the healing process as well as the main protein that plays an important role in compiling

extracellular matrix components. In extracellular matrix deposition, collagen synthesis is propagated by growth factors and cytokines with cell expression, one of which is Fibroblast Growth Factor (FGF).[3,4,5]

Fibroblasts are specific cells that differentiate from mesenchymal cells resting in connective tissue. After being stimulated by macrophages, platelet-derived cytokines, and growth factors, fibroblasts undergo replication and proliferation. Fibroblasts can initiate replication through an autocrine process that releases FGF. FGF is a representative growth factor that shows potential effects on tissue repair and regeneration. FGF exhibits multiple functions in binding and activation with FGF receptors (FGFRs), and the main signal through the stimulation of FGFRs is the RAS/MAP kinase pathway.[6,7]

FGF-1 and 2, also known as acidic and basic FGFs, are produced by inflammatory cells, vascular endothelial cells, fibroblasts, and keratinocytes. Both play a role in Re-epithelialization, the process of angiogenesis, and the formation of granulation tissue.[8] In experiments conducted on animals, it was found that the disruption of the FGF signal will result in defects in the urinary tract.[5] In another study, using a tube-formed Dry Amniotic Membrane Composite that had previously been implanted with mesenchymal stem cells from rabbit fat tissue, it was said that the highest cell expression of FGF in the scaffold seeding stem cell group was formed.[9]

This study makes researcher interested in the expression of FGF in urethral defects using FD-AM graft method compared to primary repair.

2. Methods and Material

2.1. Study Design

This study was conducted at Laboratorium of Pharmacology of Airlangga University, Department of Pathology Anatomy of Airlangga University, and Department of Biochemistry of Brawijaya University, Malang. This study is an experimental study using the randomization technique in sample selection and has received an ethical assessment and approval from the Animal Care and Use Committee for using experimental animal.

2.2. Study Population

Total sample of 36 New Zealand white rabbits (Oryctolagus sp.) were obtained from Farma Veterinary Center, after a homogeneous test, the samples were divided into a control group and a treatment group (18 rabbits each).

2.3. Production of FD-AM

Fresh amniotic membranes were taken from mothers with elective cesarean delivery using aseptic instruments and techniques. The amniotic membrane was washed with saline containing streptomycin 50 g/ml, penicillin 50 g/ml, then the inside was bluntly separated from the chorion. The amniotic membrane was immersed in BEM solution as a transport medium and stored at 4^{o}C for extraction before 24 hours. Next, it was sent to the laboratory in a sterile with dry ice.

The amniotic membrane was cleaned and washed with antibiotics as above, then dried on drying paper, and the wet weight was weighed and the volume was measured. After that, the amniotic membrane was transferred to a deep freezer to prepare for the freeze-drying process for 24-48 hours at -800C. The next process goes to the freeze dryer process until the drying is optimal (for 7-8 hours) and then it is packaged through laminar airflow and chemically sterilized using J radiation (25kGy).

2.4. Samples Preparation

After being adapted for seven days, the rabbits underwent urethroplasty under general anesthesia using ketamine 20-40 mg/kg body weight intramuscularly. Each rabbit was shaved in the lower abdomen, then disinfected with 10% povidone-iodine and covered with a sterile

drape. Both groups were fitted with a 6fr foley catheter as a splint. The skin of the penis at the incision extended longitudinally to a length of 3 centimeters. The incision is deepened until it reaches the urethral wall. The urethral wall was cut transversely one centimeter, 10 mm from the meatus with a surgical blade no 15. For the treatment group, the defect was closed with a patch of 6 folds FD-AM with a size of 1x1 cm on the ventral surface of the urethral mucosa with PDS 6/0 sutures using the interrupted technique. Meanwhile, the control group was closed using primary repair. Furthermore, both groups were fixed on the catheter using 3/0 silk thread which was sewn to the glans penis. While the raised part of the catheter was cut, so the rabbit could not remove the catheter.

The urethral tube is retained for seven days, then removed. The sutured wound on the skin of the penis was not closed. On the 28th day postoperative, a small specimen was taken from the proximal part of the operating area in each rabbit in parallel transverse sections with a thickness of 2mm by cutting the urethra at an angle corresponding to the longitudinal axis. Where previously, the treated side of the urethra was marked with black ink to distinguish it from the untreated side. Then put in 10% formalin and sent for histological examination.

2.5. Immunohistochemical Examination

The specimens were fixed in 4% buffered formaldehyde for 48 hours at 4ºC and followed by immersion in 0.1M/L Phosphate Buffer Saline before fixation with paraffin. Examination of immunocompetent cells expressing FGF was carried out using immunohistochemical techniques which were assessed and counted through five fields of view with 400X magnification. FGF-2 expression was identified by the presence of stained brown granules in the cytoplasm of tumor cells. Expression in the epithelium, endothelial cells, and stroma were analyzed, grading for the percentage of positive areas was done as follows: <10% = 0.10-25% = 1.25–50% = 2.50–75% =3, >75 = 4. To evaluate the intensity, the grading was carried out as follows: 0 = none, 1 = mild, 2 = moderate, 3 = strong staining. The final score was determined by multiplying the percentage score (0-4) with the intensity score (0-3). 0–4 for negative scores and 5–12 for positive scores. The reading of the preparation was done by binding, where the reader did not know the code of the preparation for the treatment group and the control group.

2.6. Observation of Clinical Symptom of the Wound

On the 28th day, which is the maturation period at the end of the wound healing process in rabbits, the penile skin is opened and the urethra is freed from the surrounding tissue to see if there is a fistula formation or no fistula or no urethra at all. As ordinal data, scores were used for the following classifications: Grade 0: open wound/dehiscence, Grade 1: cutaneous urethral fistula formed, and Grade 2: urethra without fistula formed.

2.7. Data Analysis

Study data processing and analysis were conducted through computerization using SPSS ver. 21 program. Correlation analysis between 2 groups of ordinal data was performed.

3. Result

The results of the comparison test of FGF in the control and treatment groups with the Mann Whitney test obtained p = 0.00 which means the FGF in the treatment group was significantly higher than the control group. In the control group, the smallest FGF value is 4 and the largest value is 9, with an average value of 6.5, wherein the treatment group the smallest FGF value was 9 and the largest was 12, with an average value of 11.61.

While the results of the analysis of the relationship between FGF and wound healing using the Spearman test was found that in the control group, p-value > 0.05 (0.757), while in the treatment group, p-value > 0.05 (0.670). So it can be concluded that statistically, there is no relationship between FGF and wound healing in both groups.

4. Discussion

This study is a study on the use of FD-AM in experimental animals, New Zealand white rabbits (*Oryctolagus sp*). FD-AM is a material that is widely used to help stimulate the healing process. The amniotic membrane has long been known as a biological dressing in wounds because it can stimulate wound healing such as increasing the formation of collagen tissue, accelerating epithelialization, and the formation of new blood vessels.[1]

The study evaluated the amount of FGF on the 28th day by comparing it into 2 groups, namely the primary repair of urethral defects without using FD-AM (control) and groups using FD-AM (treatment). In the control group, the values obtained were the smallest FGF was 4 and the largest FGF value was 9, with an average value of 6.5, wherein the treatment group the smallest FGF value was 9 and the largest FGF value was the maximum value of 12 with an average value of 11.61.

In nominal terms, the amount of FGF was higher in the group with dry amniotic membranes in urethral defects when compared to the amount of FGF in reconstructed urethral defects with primary repair. Then in these two groups, the number of FGF was tested statistically. With the Mann Whitney test, p = 0.00 was obtained where a significant value was obtained. This means it can be concluded that the number of fibroblast growth factors in the treatment group using FD-AM was more than the control group with a primary urethral repair. On the 28th day, FD-AM has been absorbed and new epithelial cells have grown so that the urethra appears intact, even a lot of neovascularization was found on examination under a microscope. The image with 400X magnification clearly shows the growth of the epithelium with several FGF-expressing cells (see Figure 1).

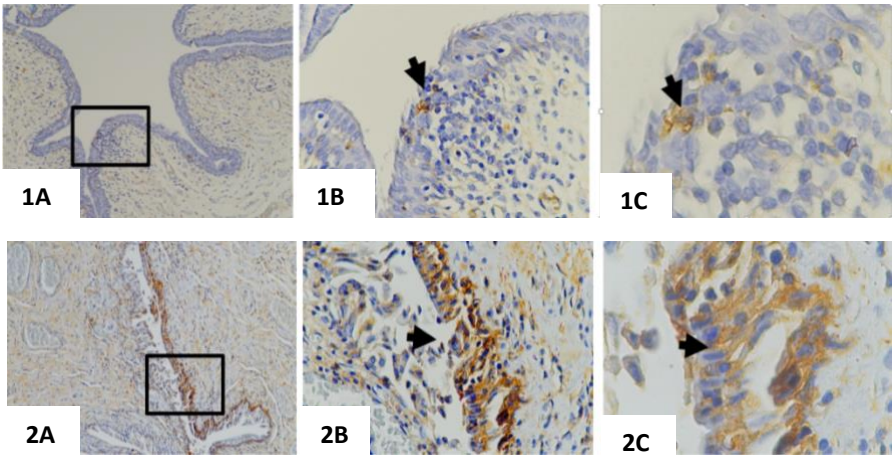

Figure 1. Histopathological features of FGF in the control group (**1A**) (**1B**) (**1C**). Histopathological features of FGF in the treatment group (**2A**) (**2B**) (**2C**)

From the conclusion above, it can be concluded that the hypothesis about the expression of FGF in urethral defects reconstructed using FD-AM is higher than that of primary repair is acceptable. There is following research conducted by Arifianto (2016) who found that FD-AM applied to the wound site showed an increase in FGF when compared to primary repair which was also carried out in an experiment with rabbit gingiva, with faster wound healing.[10] The number of fibroblast cells also increased in wound healing using dry amniotic membranes in the rabbit trachea when compared to primary repair in an experiment conducted by Corputty (2020) which indicated an accelerated wound healing process in the rabbit trachea.[11,12] In addition, in an experiment by Hariastawa (2020), it was also said that FGF plays a very significant role in wound healing and induces the formation of collagen and the growth of

urethral epithelial cells by adding dry amniotic membranes seeding mesenchymal stem cells as a graft for urethral reconstruction.[9] For an experiment to calculate the amount of FGF in urethral wounds with a dry patch of amniotic membrane, this experiment is the first time this experiment has been carried out.[11]

The study also evaluated wound healing by comparing it into 2 groups, namely the primary repair of urethral defects without using FD-AM (control) and a group using FD-AM (treatment). From the study, the control group obtained 4 rabbits (22.2%) who had fistulas on wound evaluation, while 14 rabbits (77.8%) had their urethras healed well without any fistulas or dehiscence. In the treatment group of 18 rabbits, 1 rabbit (5.6%) had fistula while the rest recovered completely. Nominally, the number of urethral wounds with fistulae was less in the treatment group than in the control group. However, from the test results obtained p-value >0.05 (0.154), so it can be concluded that there is no statistically significant difference in the comparison of the 2 groups, namely the primary repair of urethral defects (without using FD-AM) compared to the group using FD-AM. Where there is almost the same experiment conducted by Sugianto (2017) where it is said that there is no difference in the leakage rate in rabbit urethral defects reconstructed using FD-AM compared to primary repair.[13] What might have influenced the results was the sampling time in which the researchers took rabbit urothelial tissue in the maturation phase, where the greatest effect of fibroblasts was on the proliferative phase of wound healing.

According to Werner and Grose, the amniotic membrane plays a role in forming the extracellular matrix and adding fibroblasts which will gradually be replaced by collagen in the maturation phase which will then link the wound edges.[8] For the occurrence of fistulas in this experiment, it is also not known clearly because the researchers did not perform urothelial tissue deposition and investigated the deficiency of other growth factors besides the amount of FGF. However, as we know, infection and ischemia are the two most important factors that cause fistulas.[1]

5. Conclusion

There was a difference in the amount of FGF expression in the primary repair of urethral defects (without using FD-AM) compared to the group using it. Where the amount of FGF in the group using FD-AM was more than the primary repair group. No significant association was found between increased FGF and wound healing in this study, however, dry human amniotic membranes can be considered as a substitute for primary repair of urethral reconstruction in clinical applications. This study has limitations where the researcher did not perform a histopathological examination to compare the number of fibroblasts in each phase of wound healing. In addition, there is no standard for patching the urethral suturing technique, which certainly requires a lot of other research in the future. Researchers hope that further research will be carried out by considering various urethral reconstruction techniques by patch.

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There is no conflict of interest in this study.

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