Effect of Amniotic Membrane-Derived Mesenchymal Stem Cells on TNF-α Expression and Inflammatory Cells Infiltration during Vesicovaginal Fistule Repair Healing Process

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Abstract

Background: Vesicovaginal fistula (VVF) causes high morbidity in women, affecting both physical and psychological condition. Until now, surgery is the mainstay treatment for this condition. However, prolonged exposure during inflammatory phase after surgery is still a problem in the healing process. TNF-α as potent pro-inflammatory cytokine plays an important role by attracting inflammatory cells to wound tissue. Amniotic membrane is the source for mesenchymal stem cells that had anti-inflammatory and imunomodulatory effect. This study aims to evaluate the effect of Amniotic Membrane-Derived Mesenchymal Stem Cells (AMMSC) on TNF-α expression and inflammatory cell infiltration during VVF repair healing process in New Zealand White (NZW) rabbit model.

Method: This study was an experimental study with randomized posttest only control group design. Twenty-seven NZW rabbit as VVF model was used in this study, randomly divided into 3 different treatment groups after underwent surgical treatment (no treatment <C group>, treated with freeze-dried amniotic membrane <T1 group>, and treated with freeze-dried amniotic membrane that seeded with AMMSC <T2 group>). Evaluation was done 7 days after treatment. TNF-α expression was evaluated semiquantitatively using modified Remmele-Stegner scale. Inflammatory cell infiltration was evaluated using modified Klopfleisch method.

Results: Mean TNF-α expression between C, T1, and T2 group were significantly different (8.5 ± 1.6; 7.1 ± 1.2; 1.6 ± 1.2 respectively, $p < 0.001$). Median inflammatory cell infiltration between C, T1, and T2 group were significantly different (3.0; 2.0; 1.0 respectively, $p < 0.001$).

Conclusion: AMMSC significantly reduced TNF-α expression and inflammatory cells infiltration during VVF repair healing process.

Keywords*: vesicovaginal fistule, amniotic membrane mesenchymal stem cells, TNF-α, macrophage, neutrophil*

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Introduction

Vesicovaginal fistula (VVF) causes high morbidity in women, affecting both physical and psychological condition. Quick and accurate diagnosis followed by timely repair is essential to the successful management of these cases. Surgery with primary suturing are still the treatment of choice despite the challenge in healing process and to reduce the recurrence rate [1]. The key to achieve satisfactory surgical outcome for VVF is by

having a good visualization of the surgical field, adequate necrotic tissue dissection, tissue approximation, and the drainage of urine. Surgical access could be transvaginal or transabdominal, depending on the location, size, and profile of the area around the fistula. Another important factor that affect the success of the surgery is the surgeon's experience [2].

Failure in VFF healing after fistula surgery is complex and influenced by the severity of the surrounding tissue. Recent systematic review found that the success rate in VFF healing after surgery in developed countries are 94.6%, and 80.4% in patients with cervical malignancy after radiation. Recurrence was reported three months after the surgery with an average failure on day 25. In developing countries, VFF recurrence occurred around 41% [3] L. M.</author><author>Rothschild, J. G.</author></authors></contributors><titles><title>Complications and Long-Term Sequelae of Bladder Fistula Repair</title><secondary-title>Current Bladder Dysfunction Reports</secondary-title></titles><periodical><full-title>Current Bladder Dysfunction Reports</full-title></periodical><pages>317-24</pages><volume>11</volume><number>4</number><dates><year>2016</year></dates><urls></urls></record></Cite></EndNote>. Healing wounds in urogynaecology system require a longer process than in skin because of the influence of spongiosum tissue around the wound and pro-inflammatory cytokine that alter the cell migration. The potent pro-inflammatory cytokine which plays a major role is tumor necrosis factor alpha (TNF-α). TNF-α will stimulate the inflammatory process, attract neutrophils and macrophages to clean up debris around the wound. Once this phase is passed for 4 days, then progressed to proliferative phase. When the inflammatory phase occurs for longer period, it will disrupt and impede the wound healing process [4].

Urogynaecology tissue engineering had promising result as a potential therapy by reconstructing tissue engineering and tissue regeneration using a combination of biomaterials, stem cells and other biomolecules. Stem cells have the property of self-renewal, differentiation and immunomodulation. It can be given as monotherapy or combined with tissue framework (scaffold) serves to support the cell to attach and differentiate. Local injection of stem cella in experimental animals showed good results. This gives hope to the future in the management of urogynaecology cases [4].

Human amnion is one source of biomaterials and stem cells. As a biomaterial, amnion can be used directly to close the defect wounds or as scaffold. The one used is dried amnion as a source of stem cells and resemble mesenchymal stem cell (MSCs). The amnion has low immunogenicity properties, antimicrobial and anti-inflammatory effect [5]. It is able to express growth factor as well as proteins such as collagen, glycoproteins, integrins, and lamellar body that helps cells to grow and proliferate. The additional effect is immunomodulation of amniotic stem cells and anti-inflammatory [6]. This study aims to evaluate the effect of dry amniotic and seeding amniotic membrane mesenchymal stem cell (AMMSC) on the expression of TNF-α and inflammatory cell infiltration in VVF using animal model.

Method

This study was an experimental analytic study with randomized posttest only control group design.This study was conducted at the Tropical Disease Center Airlangga, Surabaya on March-May 2017. Samples in this study was female New Zealand White (NZW) rabbits with inclusion criteria as follows: weigh 3-4.5 kg, aged 3-5 months, and without birth defect or scar. Dropout criteria for this study were rabbits whose died during the study period.

There were 27 rabbits used in this study. All rabbits were designed to had VVF by making a 5 mm defect on the walls of the vagina and the urinary bladder via laparotomy. Vaginal and urinary bladder wall was then sewn with thread SAFIL 4-0 HR 22 (BI Braun, Tutlingen, Germany), then the defect is maintained by Naso Gastric Tube (NGT) 16F for 3 weeks. Prior to surgical procedure, subjects were under anesthesia using ketamine 25-40 mg/kg and azepromazine 0.25-1 mg / kg intramuscular. After the procedure, tolfenamic acid 10 mg / kg / day were given for post-surgical pain reliever.

After 3 weeks, subjects were divided into 3 groups using simple random sampling, namely control (C) group, treatment 1 (T1) group, and treatment 2 (T2) group. C group underwent laparotomy and the VVF was treated with simple interrupted suture at the vaginal wall and bladder wall using a thread SAFIL 4-0. T1 group underwent the same procedure as C group and receive additional treatment of dry amniotic 1x1 cm placed between the vagina and bladder walls that was sewn at each corner using a thread SAFIL 4-0. T2 group underwent the same procedure as C group and receive

additional treatment of dry amniotic size 1x1cm seeding with AMMSC placed between the vagina and bladder walls that was sewn at each corner using a thread SAFIL 4-0.

Seven days after the treatment, all subjects underwent another lapartomoy procedure to collect sample from the VVF with the size of 1x1 cm for inflammatory cell infiltration and TNF-α evaluation using histological examination. The sample was then washed with 0.9% NaCl fluid twice before stored in the container with 10% buffer formalin for 48 hours. Hematoxilin-eosin staining was done to evaluate the inflammatory cell infiltration (neutrophil and macrophage), while immunohistochemistry staining was done to evaluate the expression of TNF-α. The number of neutrophil and macrophage cells was evaluated under the microscope and were scored using modified Klopfleisch method [7]. Expression of TNF-α were evaluated semiquantitatively using modified Remmele-Stegner Scale based on the intensity of expression and the number of cells/tissue area positively stained [8].

Acquired data was analyzed using SPSS version 17.0 (SPSS, Inc., Chicago IL). Shappiro-Wilk test was used to analyze the normality of the data. Kruskall Wallis test was used to compare the bodyweight and inflammatory cell infiltration between 3 groups. Mann-whitney test was used to compare the inflammatory cell infiltration between 2 group. ANOVA test was used to compare the TNF-α expression between 3 groups. Post hoc LSD test was used to compare the TNF-α expression between 2 group. A $p < 0.05$ was considered statistically significant.

Result

There were 27 NZW rabbits with no dropout included in this study. Bodyweight of the rabbits used in this study ranged from 3200 to 3700 grams. After the rabbits had been randomly grouped into 3 groups, normality test showed that the bodyweight in C group, T1 group, and T2 group was normally distributed ($p = 0.172$; 0.296; and 0.072, respectively). There was no significant difference between bodyweight of C group, T1 group, and T2 group (3366.67 ± 173.2 gram, 3388.89 ± 145.3 gram, and 3455.56 ± 159 respectively; $p = 0.461$).

TNF-α expression was evaluated semiquantitavely using modified Remmele method. There were significant differences between C group, T1 group, and T2 group ($p < 0.001$). Post-hoc LSD test showed significant differences between C group and T1 group ($p = 0.029$), C group and T2 group ($p < 0.001$), and between T1 group and T2 group ($p < 0.001$) (table 1). Inflammatory cell infiltration was evaluated using modified Klopfleisch method. The neutrophils and macrophages in each group was observed under the microscope (Figure 1). There was a significant score difference between groups ($p < 0.001$). Kruskall walls test showed significant score difference between C group and T1 group ($p = 0.007$), C group and T2 group ($p = 0.001$), and between T1 and T2 group ($p = 0.022$) (table 2).

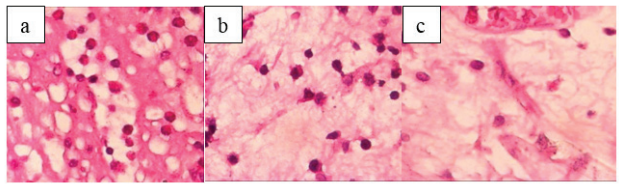

Figure 1. Lamina propria of the vagina infiltrated by inflammatory cells (neutrophils and macrophages) observed under the microscope with 100x magnification in: a) group C, b) group T1, c) group T2.

Table 1. TNF-α expression between groups

Group	Mean ± SD	p-value
C	8.5 ± 1.6a†	< 0.001*#
T1	7.1 ± 1.2b†	
T2	1.6 ± 1.2c†	

*P < 0.05 was considered statistically significant

#One-way ANOVA test was used

†LSD test was used. Values in a column with different superscripts were significantly different (P < 0.05) from each other

Table 2. Inflammatory Cell Infiltration Between Groups

Group	Median	p-value
C	3.0 a‡	< 0.001*#
T1	2.0 b‡	
T2	1.0 c‡	

* P < 0.05 was considered statistically significant

Kruskall Walls test was used

‡ Mann Whitney test was used. Values in a column with different superscripts were significantly different (P

< 0.05) from each other

Discussion

There are significant differences between C group, T1 group, and T2 group in this study. TNF-α expression is highest in C group, and the expression is lower in T1 group where the subjects received an additional treatment of dry amniotic. In T2 group where the subjects received an additional treatment of dry amniotic seeding with AMMSCs, the expression of TNF-α was the lowest compare to C group or T1 group. This finding showed that seeding amniotic stem cells can significantly decrease TNF-α as pro-inflammatory cytokines. Previous study that compared between the amnion and amniotic administration with seeding adipose stem cells on healing of rat skin found that in group received stem cells treatment had accelerated wound healing with minimal inflammation [6].

Amniotic membrane has anti-inflammatory effects and helps the wound healing process. Previous study proved its effectivity in the cornea reconstruction and skin wound healing [5]. Other study which compared the use of dry amniotic scaffold hyaluronic acid based on case repair tendon found a significant reduction in TNF-α expression on the seventh day [9]. Another study found that in rabbits given mesh made from propilene compared with added amnion, the level of inflammation is significantly decreasesd when receive the amnion treatment [10].

Amnion is part of the fetus during pregnancy which express nonclassic Human Leukocyte Antigen G (HLA-G) (class Ib antigen). HLA-G polymorphism of this type is lower than the mother's antigen class. Because of the low immunogenicity, maternal immune system does not attack the fetus. There are two proposed mechanisms of how the amniotic membrane has a low immunogenicity. The first one is that the HLA-G plays a role in reducing the activity of lymphocytes and dendritic cell when it binds to receptors inhibitor. The second mechanism is when HLA-G recognized by CD8 T cells and activated, CD8 would bind to HLA-G and will have the immunosuppression function [11]. This mechanism might explain why there has been no rejection to the seeding amniotic stem cell.

In our finding, there are significant differences in inflammatory cell infiltration between groups. In C group where the subjects did not receive additional treatment other than surgical treatment, the inflammatory cell infiltration was significantly higher compare to the other groups. In T1 group where the subjects receive additional treatment of dry amniotic compared to T2 group where the subject receive additional treatment of dry amniotic seeding with AMMSCs. Previous study in the case of duodenum repair showed that suture plus amnion patch compared to primary suture alone had a lower inflammatory infiltration of histopathologic score. Anti-inflammatory mechanism of amniotic believed to be a factor in reducing the response of inflammatory cells in the tissue. In addition, amnion contain growth factors and multipotent cells that help the process of angiogenesis and fibroplasia [12].

Mesenchymal stem cells can regulate the proliferation, activation, and as an effector for T cells, dendritic cells, macrophages, NK cells, and neutrophils. Stem cells that are affected will polarize the inflammatory environment and convert M1 into M2 macrophages that have anti-inflammatory properties. M2 macrophage phagocytic activity and the secretion of proinflammatory cytokines such as IL-12 and TNF-α is low. Factors affecting the intermediary of M2 is PGE2, TNF-α stimulated gene 6 (TSG-6), and IDO. In the acute inflammatory phase, mesenchymal stem cells protect neutrophils from apoptosis through activation of TLR3. In chronic inflammatory phase, the effect of IFN-γ reduction will reduce the migration of neutrophils to the network to prevent further damage [13, 14].

Macrophages control lifespan of neutrophils to secrete receptor death ligands as Fas ligand (FasL), TNF-α and TRAIL. When FasL is secreted at low concentration, it will extend the lifespan of neutrophils and increase infiltration into the wound tissue. TNF-α delay the apoptosis of neutrophil through transcription factor NF-κβ in order to improve the tissue survival [15]. TGF-β thought to regulate the activity and function of neutrophils. TGF-β inhibition will increase the infiltration of neutrophils in tissues. Analogous to the M1 and M2 macrophage polarization, polarize TGF-β phenotype of neutrophils [16]. Provision of amniotic stem cells will decrease the expression of TNF-α in wound tissue, where low TNF-α will not suppress the expression of TGF-β. This is in accordance with the histopathologic evaluation of T2 groups in our study where there are decreased neutrophil infiltration compared to T1 and control.

Conlussion

Amniotic membrane could reduce TNF-α expression and inflammatory cell infiltration during the VVF repair healing process in NZW rabbit model. Seeding the membrane with AMMSC enhance the reduction effect more significantly.

Conflict of Interest: There is no conflict of interest

Source of Funding: This study is self-funded

Ethical Clearance: This study was ethically approved by Ethics Committee of Faculty of Medicine Universitas Airlangga (Ethical Clearance Number: 171/EC/KEPK/FKUA/2017) before conducting the study. All experiments were performed in accordance with relevant regulations.

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