Original Article

Effect of Umbilical Cord Mesenchymal Stem Cells on Vascular Maturation in Rabbit Arteriovenous Fistula (AVF) Model Assessed using Doppler Ultrasonography

Iman Akbar Hasibuan,* Yopie Afriandi Habibie, Erwin,*** Jufriady Ismi,**** Muhammad Yusuf,***** Nanda Yulian Syah*****

**Resident of Surgery, Faculty of Medicine, University of Syiah Kuala, dr. Zainoel Abidin Hospital, Banda Aceh, Indonesia*
***Division of Cardiothoracic Surgery, Faculty of Medicine, Syiah Kuala University, dr. Zainoel Abidin Hospital, Banda Aceh, Indonesia*
****Faculty of Veterinary Medicine, Syiah Kuala University, Banda Aceh, Indonesia*
*****Division of Urology, Faculty of Medicine, University of Syiah Kuala, dr. Zainoel Abidin Hospital Banda Aceh, Indonesia*
******Division of Digestive Surgery, Faculty of Medicine, Syiah Kuala University, dr. Zainoel Abidin Hospital, Banda Aceh, Indonesia*

Abstract

Introduction: *The need for arterio-venous fistula (AVF) procedures continues to increase along with the rising incidence of chronic kidney disease. However, AVF maturation remains a challenge in clinical applications due to its susceptibility to various factors with complex mechanisms. Mesenchymal stem cells (MSCSs) have the potential to stimulate tissue regeneration, particularly in vascular injuries. This study aims to assess the effect of in situ and intravascular MSCSs administration on AVF maturation.*
Methods: *This study employed an experimental design utilizing an animal model, specifically the Lepus domestica rabbit. Vascular maturation was assessed through parameters such as diameter, hyperplasia, and flow using Doppler Ultrasonography (USG) over a 14-days period. The production of stem cells was conducted using fluids and umbilical cord membranes. Statistically analysis involved one-way ANOVA and Kruskal Wallis test, followed by a post Hoc, with a confidence level of 95%.*
Results: *A total of 28 Lepus domesticas were utilized, distributed across three groups: P1-negative control (n=9), P2-MSCSs in situ (n=9), and P3-MSCSs intravenous (n=10). Group P2 showed the widest vascular diameter ($p<0.001$) and the least formation of hyperplasia ($p=0.014$), with mean values of 4.5 mm and 1.2 mm, respectively. The P3 group demonstrated the fastest flow compared to the other groups ($p=0.02$), with an average flow of 144.6 mL/min.*
Conclusion: *The administration of MSCSs in situ enhances vascular maturation following AVF procedures by increasing diameter size and reducing the formation of vascular hyperplasia.*

Keywords: *Arterio-venous fistula, Mesenchymal stem cells, Vascular maturation.*

Korespondensi: **Iman Akbar Hasibuan**
E-mail: *imanakbar030911@gmail.com*

Pengaruh Pemberian Sel Punca Mesenkimal Tali Pusat Terhadap Maturasi Vaskular Berdasarkan Pemeriksaan Ultrasonografi Doppler pada Kelinci Lokal Model Arteriovenosus Fistula (AVF)

Iman Akbar Hasibuan, Yopie Afriandi Habibie,** Erwin,*** Jufriady Ismi,**** Muhammad Yusuf,***** Nanda Yulian Syah****

**Residen Bedah Fakultas Kedokteran Universitas Syiah Kuala/Rumah Sakit dr. Zainoel Abidin, Banda Aceh, Indonesia*
***Divisi Bedah Thorak Kardiovaskular Fakultas Kedokteran Universitas Syiah Kuala/Rumah Sakit dr. Zainoel Abidin, Banda Aceh, Indonesia*
****Fakultas Kedokteran Hewan, Universitas Syiah Kuala, Banda Aceh, Indonesia*
*****Divisi Urologi, Fakultas Kedokteran Universitas Syiah Kuala/Rumah Sakit dr. Zainoel Abidin, Banda Aceh, Indonesia*
******Divisi Bedah Digestive Fakultas Kedokteran Universitas Syiah Kuala/ Rumah Sakit dr. Zainoel Abidin, Banda Aceh, Indonesia*

Abstrak

Pendahuluan: *Kebutuhan prosedur arterio-venous fistula (AVF) terus mengalami peningkatan seiring meningkatkan kasus penyakit ginjal kronik. Kendati demikian, maturasi AVF masih menjadi tantangan dalam praktik klinis karena dipengaruhi oleh berbagai faktor dengan mekanisme yang kompleks. Sel punca mesenkimal (mesenchymal stem cells MSCSs) memiliki potensi dalam merangsang regenerasi jaringan, khususnya pada cedera vaskular. Penelitian ini bertujuan untuk menilai pengaruh pemberian MSCSs in situ dan intravaskular terhadap maturasi AVF.*
Metode: *Penelitian ini merupakan studi eksperimental menggunakan model hewan coba kelinci Lepus domestica. Maturasi vaskular diukur dengan menggunakan parameter diameter, hiperplasia, dan aliran vaskular dengan menggunakan ultrasonografi Doppler selama 14 hari. Pembuatan sel punca dilakukan menggunakan cairan dan membran tali pusat. Analisis statistik meliputi uji one-way ANOVA dan Kruskal Wallis, dilanjutkan dengan uji post Hoc dengan tingkat kepercayaan 95%.*
Hasil: *Sebanyak 28 Lepus domestica digunakan dalam studi ini dan terbagi dalam 3 kelompok: P1-kontrol negatif (n=9), P2-MSCSs in situ (n=9) dan P3-MSCSs intravena (n=10). Kelompok P2 menunjukkan diameter vaskular paling besar (p<0,001) dan pembentukan hiperplasia paling minimal (p=0,014) dengan nilai rerata secara berurutan 4,5 mm dan 1,2 mm. Kelompok P3 menunjukkan aliran yang paling cepat dibandingkan kelompok lainnya (p=0,02) dengan rerata kecepatan 144,6 mL/menit.*
Kesimpulan: *Pemberian MSCSs in situ dapat meningkatkan maturasi vaskular pascaprosedur AVF dengan meningkatkan ukuran diameter vaskular dan menurunkan pembentukan hiperplasia vaskular.*

Kata kunci: *Arterio-venous fistula, Sel punca mesenkimal, Maturasi vaskular pandemic.*

Introduction

Chronic kidney disease (CKD) is one of the leading causes of death and disability worldwide. This condition requires therapy in the form of hemodialysis which requires vascular access. Based on data from the Indonesian Renal Registry in 2018, there was an increase in CKD cases from 2007. Treatment for end-stage renal disease (ESRD) patients in the United States in 2017 consisted of hemodialysis (62.7%), peritoneal dialysis (7.1%), and kidney transplantation (29.9%). As many as 80% of ESRD patients use intravenous catheter vascular access at the start of hemodialysis. Maintaining vascular access patency for patients with (ESRD) is crucial because it allows optimal hemodialysis action. Arterio-venous fistula (AVF) is the preferred and permanent vascular access; however, its patent rate in

the first year is estimated to be only 62%. The problem of low AVF patent rates in the first year needs to be addressed to prevent repeated AVF surgeries and increase AVF patent rates as permanent vascular access.[1-3]

The immature AVF is believed to arise from inadequate outward remodeling and premature vascular maturation. The primary cause of AVF failure is stenosis. Various factors contribute to the vascular maturation process, including hypoxia, shear stress, oxidative stress, and inflammation. Reduced blood flow and the presence of shear stress inhibit vascular vasodilation and promote vascular immaturity. This mechanism occurs due to endothelial activation, low levels of nitric oxide (NO), and the release of inflammatory mediators that include vascular stenosis. Vascular stenosis can trigger hemostasis and lead to thrombosis, causing fistula to remain immature or fail.[4,5]

Due to the incidence of vascular immaturity in AVF, mesenchymal stem cells (MSCSs) have been considered a promising therapy for vascular injury. MSCSs therapy has been utillized in various pathological conditions to stimulate tissue regeneration. Depending on the specific milieu, MSCSs can differentiate into several cell types. Previous studies have reported the benefits of MSCSs in cases of calvarial defects, Crohn's disease, critical limb ischemia, and multiple sclerosis, as well as in improving wound healing.[6]

Criteria for the diagnosing AVF using Doppler ultrasound and duplex ultra-sound include low- and high-resistance flow in the supplying artery, high-velocity arterial waveforms in the draining veins, and high-velocity turbulent flow spectra at the junction of the arteries and veins. Ultrasonography can also detect the failure of blood vessels to mature. Doppler ultrasound serves as a valuable tool for identifying obstructive issues. It has consistently demonstrated accuracy and reproducibility in diagnosing access complications when compared to access angiography. Abnormal duplex flow studies exhibit greater sensitivity in detecting AVF failure or the necessity for reintervention than physical examination alone. Abnormal duplex are more correlated with the need for re-intervention to achieve maturation than findings from physical examination.[7]

Based on the findings from these studies, it is evident that the demand for AVF as access for hemodialysis is on the rise. However, the failure rate for AVF maturity is also significantly high. Therefore, further research is required to investigate the impact of umbilical cord MSCSs on reducing the incidence of vascular immaturity. This investigation is aimed to determine the effect of umbilical MSCSs on vascular maturity and AVF patency based on Doppler ultrasound. The study will be carried out in an AVF rabbit model.

Method

This experimental research was conducted using factorial randomized block design approach (RAKF). Samples were Lepus domestic rabbit models. We divided samples into three groups: negative control (without treatment), treatment group 1 (*in situ* MSCSs administration), and treatment group 2 (intravenous MSCSs administration).

Vascular maturation was measured by parameters of diameter, hyperplasia, and flow using Doppler Ultrasonography (Mindray® DP-10) for 14 days. The manufacture of stem cells was carried out using fluids and umbilical cord membranes. Statistical analysis involved one-way ANOVA and Kruskal-Wallis analysis, followed by a post hoc test, with a confidence level of 95%. This research has received approval for experimental animal ethics clearance from the Faculty of Veterinary, Universitas Syiah Kuala, Aceh (certificate of ethics: Ref 207/KEPH/IV/2023).

AVF Model Procedure in Rabbit

The procedure for creating AVF models in experimental animal models was conducted through the following steps. This procedure was carried out by a Thoracic Cardiovascular Surgeon and a veterinarian. In the first step, local rabbits (*Lepus domestica*) were anesthetized with ketamine at 30 mg/kg IM in the left quadriceps femoris muscle, and the rabbits breathed spontaneously during the procedure. Aseptic and antiseptic measures were then implemented in the right coli area. We made an incision along the common carotid artery, then identified the left carotid artery and left jugular vein. We performed end-to-side anastomosis on the arteries and veins in the left colli area using countinous suture with 8-0 prolene thread and evaluate until a thrill was palpable.

Isolation and Culture of Umbilical Cord Mesenchymal Stem Cells

Stem cells were obtained from the umbilical cord of a human donor. Umbilical cord MSCSs were obtained from HayandraLab®, Jakarta. The method, known as the H-Remedy method (patent application no. P00201603083), was developed by HayandraLab, as a refinement of the previous method. Lipoaspirate

(approximately 15-600 mL) was digested by H-Remedy enzyme and incubated for 1 hour at 37 °C, 300 rpm. After incubation, the digested lipoaspirate was added to Dulbecco's low-glucose (1g/L) modified Eagle's medium (DMEM) containing 4 mML glutamine (Gibco®, USA) to inactivate the enzyme, followed by centrifugation for 5 min at 600 ×g. A combination of low glucose DMEM containing 4 mM L-glutamine supplemented with 10% FBS (Gibco®), 1% antibiotic-antimycotic solution (10,000 units/mL penicillin, 10,000 g/mL streptomycin, and 25 g/mL Amphotericin B) (all from Gibco®), and 0.05 ng/mL L-ascorbic acid were used as culture media. The culture medium was replaced every 2-3 days with fresh medium. Cells were subcultured after reaching 80% confluence. Passage 1 MSCSs were harvested and stored in saline at 4 °C for 1-3 days before use.

Interventions Placebo and Mesenchymal Stem Cells

The intervention in this research was conducted as follows: AVF model rabbits in the KP group with animal feed continuously for 2 weeks after AV fistula creation. AVF model rabbits in group P1 were fed with animal feed continuously until 2 weeks after AV fistula creation. Then, the umbilical cord MSCSs were injected *in situ* in the anastomosis area after AVF surgery. AVF model rabbits in group P2 were fed with animal feed continuously for 2 weeks after AV fistula creation. Then, the umbilical cord MSCSs were injected intravenously after AVF surgery.

Table 1. Vascular Diameter, Hyperplasia Diameter, and Blood Flow of Subjects

No	Weight (Gram)	Group*	Vascular Diameter (mm)	Hyperplasia Diameter (mm)	Blood Flow (mL/minute)
1	2100	P1	3,2	1,3	112
2	2300	P1	3,2	1,4	110
3	2230	P1	3,4	1,4	123
4	2140	P1	3,2	1,3	131
5	2200	P1	3,6	2	158
6	2410	P1	3	1,4	155
7	2300	P1	3,2	1,5	157
8	2100	P1	3	1,3	163
9	2150	P1	3,4	1,3	167
10	2330	P2	4.7	1,3	120
11	2130	P2	6.1	1,1	110
12	2320	P2	4.2	1,4	130
13	2210	P2	4.4	1,3	135
14	2150	P2	4.5	1,1	128
15	2150	P2	5.6	1,1	110
16	2250	P2	6	1,3	102
17	2400	P2	4,5	1,2	125
18	2210	P2	4,4	1,2	130
19	2330	P3	4	1,4	155
20	2400	P3	4.3	1,4	130
21	2300	P3	3,9	1,2	159
22	2200	P3	4,5	1,6	121
23	2350	P3	5	1,7	115
24	2130	P3	3,3	1,2	173
25	2210	P3	3,6	1,3	151
26	2320	P3	3,4	1,3	160
27	2200	P3	4	1,5	147
28	2350	P3	4,3	1,7	135

*P1: negative control, P2: *in situ* MSCs, P3: intravenous MSCs.

Doppler Ultrasound Examination

Doppler ultrasound examination performed by an expert veterenarian using single blind approach. Each research group underwent the following stages: we anesthetized the animal using a mixture of 0.5 cc of ketamine and 0.5 cc of xylazine. The mixture was peritoneally injected into the rabbit at 45-degrees with a dose of 0.2 cc. Eye ointment was applied to both eyes of the animal to prevent dry corneas. The lateral hair on the right thigh was shaved using a razor, and the cleanly shaved area was covered with ultrasound gel. The Doppler ultrasound probe was then attached, and the diameter, hyperplasia, and flow of the femoral vein drainage were assessed.

Results

After conducting experimental animal procedures, 28 rabbits were obtained which were distributed in each study group. All samples exhibited homogeneous body weight, with an average body weight of 2,245 grams. The evaluation of vascular maturation is assessed using three parameters: vascular diameter, vascular hyperplasia, and blood flow, as presented in the following Table 1.

Table 2. Vascular Maturation Analysis Based on AVF Draining Vein Parameters Using Doppler Ultrasound

Parameters	Median (Min-Max)			p
	P1 (n=9)	P2 (n=9)	P3 (n=10)	
Vein Diameter	3.2 (3-3.6)	4.5 (4.2-6.1)	4.0 (3.3-5.0)	<0.001*†
Vein Hyperplasia	1.4 (1.3-2)	1.2 (1.1-1.4)	1.4 (1.2-1.7)	0.014*†
Vascular Flow Velocity	141.78±22,69	121.11 ±1.33	144.6±18.69	0.02*‡

*Statistically significant.
†Kruskal Wallis test; ‡One way Analysis of variance (F=4.574).

Discussion

MSCSs have been considered as a promising therapy for vascular injury. These cells can be isolated from various sources, including adipose tissue, bone marrow, Wharton Jelly, umbilical cord blood, dental pulp, and others. MSCSs have been applied in numerous pathological conditions to stimulate tissue regeneration. Depending on the specific media used, MSCSs have the capability to differentiate into several cell types.[8,9]

Table 3. Analysis of Differences in Vascular Diameter, Vascular Hyperplasia and Vascular Flow Between Intervention Groups

Parameters	P1-P2		P1-P3		P2-P3	
	MD	p	MD	p	MD	p
Vascular diameter[1]	1.3	<0.001	0.8	<0.001	-0.5	0.006
Vascular hyperplasia[1]	-0.2	0.008	0	0.905	-0.2	0.017
Vascular flow[2]	20.66	0.024	-2.82	0.738	-23.48	0.01

[1]Mann Whitney U test, [2]Post Hoc test.

Table 2 and Table 3 below present data on the results of vascular maturation analysis. The mean vascular diameter of the group that was given *in situ* MSCSs (Group P2) had the largest diameter compared to the other groups, with an average of 4.5 mm. Statistically, a significant difference in diameter between groups was found (p<0.05), and a comparison between groups P1 and P2 showed that the difference in diameter was the most significant, with an average difference of 1.3 mm. The mean vascular hyperplasia in the group that was given *in situ* MSCSs (Group P2) showed the smallest value compared to the other groups, with an average of 1.2 mm. Statistically, it was observed that there was a significant difference in the thickness of vascular hyperplasia between groups (p=0.014) and a comparison between groups P2 and P3 showed the most significant difference in the size of hyperplasia with an average of 0.2 mm. The group that was given intravenous MSCSs showed the fastest vascular flow compared to the other groups, with an average of 144.6 mL/min, and there was a significant difference in vascular flow velocity between each group (p=0.02).

In cord blood, there are at least three different types of stem cells, including hematopoietic stem cells, MSCSs and endothelial progenitor cells. The findings of Van Pham, et al[2] show that stem cells from the umbilical cord have several advantages compared to other sources of MSCSs. Namely, their isolation does not require invasive procedures, they are abundant in quantity, and can be used off-the-

shelf.[10,11]

Stefańska K, et al[12] shows that the umbilical cord matrix, which exists in the form of a gelatinous tissue within the umbilical cord originates from extra-embryonic mesoderm tissue, can be extracted from the umbilical cord of newborns. The network is called Wharton Jelly (WJ). The connective tissue in the matrix of the umbilical cord comprises two arteries and veins. WJ is a mucosal connective tissue matrix located between the sub-amnion and perivascular, consisting of fibroblasts, collagen fibers, and proteoglycans. The cells obtained from WJ also provide a promising alternative as a source of stem cells.[12]

The findings of Yao Y, et al[13] suggest that preclinical testing in animal models is a necessary step in the development of vascular surgery. Among small animal models, rabbits possess vessels with relatively larger caliber suitable for vascular anastomosis, making them an ideal choise for preclinical test before studies in large animal models. Additionally, rabbits are considered to have a hemostatic mechanism similar to humans. In this study, the procedure for creating AVF was conducted in animal models. AVF is one of the commonly used vascular accesses, alongside arteriovenous graft (AVG) and central venous catheter (CVC).[13] The findings of Ren C, et al[14] indicate that AVF is the optimal vascular access due to its extended service life and lower incidence of cardiovascular complications.

However, approximately 30-61% of AVFs will fail in hemodialysis, either due to maturation or thrombosis. The findings of Sari NM, et al[15] and Smith GE, et al[16] indicate that several confounding variables that can influence the success of surgery and AVF patency, including age, sex and comorbidities. To control for confounding variables, the sample in this study adheres to inclusion criteria, specifically white male local rabbits, 3-4 months old, and 1,500-2,300 gram. There were also no anatomical abnormality, sign of infection, or other disease. Additionally, the research was homogenized; rabbits were weighted, rectal temperatures were measured, and they were acclimated for two weeks in individual cages measuring 120x120x160 cm. The cage temperature was set at room temperature, and every day the rabbits were given 120 grams of pellets and had access to drinking water ad libitum.[15,16]

Research by Zamboli P, et al[17] demonstrate that Doppler ultrasound plays a crucial role in identifying suitable blood vessels for creating AVF (preoperative mapping) and in the early detection of complications (surveillance). Doppler ultrasound stands out as the sole surveillance methods that enables the monitoring of AVF blood flow while concurrently investigating potential causes of compromised vascular access. This capability facilitates timely and targeted rescue interventions, ultimately prolonging the continuity of the vascular access and, consequently, the patient's life.[17]

The mean vascular diameter that recived *in situ* MSCSs (Group P2) had the largest compared to the other groups. A preclinical study by Yang K, et al[8] using rats, demonstrate that in situ administration of MSCSs at the time of creating AVF can minimize the formation of venous stenosis (VS), thereby increasing the patency of AVF. The vessels treated with MSCSs show decreased expression of the monocyte chemoattractant protein-1 (Mcp-1) gene and a significant increase in average vascular diameter compared to control vessels.[8] The findings of Cai C, et al[18] are also align with this study. They studied the effect of MSCSs on reducing stenosis after percutaneous transluminal angioplasty (PTA) in rats with AVF. The vessels treated with MSCSs after PTA have a larger vascular diameter compared to the control group.[18] Many fistulas fail to mature or develop delayed complications due to stenosis. Stenosis in AVF results from venous neointimal hyperplasia (VNH). Instead of the desired dilation of the vessel lumen, cells proliferate and migrate toward the lumen, leading to negative remodeling and eventual stenosis. Several mechanisms underlie stenosis, including hypoxia, inflammation, and shear stress that promote venous neointimal hyperplasia.[19]

The mean vascular hyperplasia in the group that was given *in situ* MSCSs (Group P2) showed the smallest value compared to the other groups. In line with these findings, a study by Barcena AJR, et al[20] also find that MSCSs administration has significant potential to increase AVF maturation. Increased AVF maturation occurs in the findings the study. This happens because MSCSs can inhibit neointimal hyperplasia (NIH) in a mouse model with CKD.[20] Vascular hyperplasia apparently also affects the size of the vascular diameter. The thickness of vascular hyperplasia is an important clinical entity in vascular surgery because it limits the long-term effectiveness of surgical and endovascular interventions. The occurrence of vascular hyperplasia can contribute to reduced vascular diameter, and vice versa, minimal hyperplasia can contribute to a larger vascular diameter.[21] Piryani AK, et al[22] suggest several factors that cause venous

neointimal hyperplasia (VNH), including shear stress, inflammation, oxidative stress, hypoxic injury to the blood vessel walls, and mechanical injury after AVF installation. Meanwhile, the findings of Cai C, et al[18] showed that MSCSs can minimize the causal factors of these VNHs because MSCSs-treated vessels experience increased vascular remodeling with decreased proinflammatory gene expression, inflammation, and fibrotic formation.

Vascular stenosis from an arteriovenous fistula is primarily the result of neointimal hyperplasia. The pathophysiology of neointimal hyperplasia consists of the deposition and proliferation of extracellular matrix, attachments, and migration of vascular smooth muscle cells (VSMCs) that exhibit abnormal healing. Both in larger and smaller hyperplasia. The group that was given intravenous MCSs showed the fastest vascular flow compared to the other groups, with an average of 144.6 mm/sec. Statistically, it was found that there was a significant difference in vascular flow velocity between each group (p=0.02). When the three groups were compared, P2 had the best effect because it demonstrated a positive diameter effect and minimal hyperplasia, though it was not optimal in terms of flow concerning a large diameter (average 4.5 mm). On the other hand, the effect of P3 is the best in terms of velocity because vascular flow is the fastest compared to the other groups.[23]

The findings of Nabila C, et al[24] obtain results that are in line with this study. The study shows a high correlation between flow velocity through the AVF and vein diameter, which flow velocity is a determinant of vein diameter. Regarding to the method of MSCSs administration used, Goncalves FC, et al[25] investigate the therapeutic effects of in situ and intravenously transplanted MSCSs in a mouse model with colitis. In contrast to this study, the findings of Goncalves FC, et al[25] indicate that intravenous therapy is a superior method for reducing colon inflammation compared to in situ therapy.

Research by Cai C, et al[18] examine the effect of MSCSs in reducing stenosis after percutaneous transluminal angioplasty (PTA) in rats with AVF. The study shows that the velocity of vascular blood flow is negatively correlated with the occurrence of stenosis, so that the lower the hyperplasia that occurs, the higher the blood flow velocity. Cai C, et al[18] also reveal that MSCS-treated vessels had significantly higher average flow velocities compared to the control group. In line with the study, other research shows that low wall shear stress is linearly related to an increase in blood flow velocity. A study by Barcena AJR, et al[20] using rats model with CKD also shows that adding MSCSs promote luminal expansion, increase blood flow, and reduce inflammatory processes underlying NIH.

Conclusions

The administration of in situ MSCSs resulted in the most significant diameter widening effect. It had the least effect on the formation of hyperplasia and the fastest vascular flow.

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