JMedSci

Journal of the Medical Sciences
(Berkala Ilmu Kedokteran)

Volume 51, Number 1, 2019; 01-10
http://dx.doi.org/10.19106/JMedSci005101201901

Correlation between micro-RNA-21 expression and inflammation cytokine in rabbits implanted with bare metal stent with the incidence of neo intimal hyperplasia

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Submited: 2018-10-08
Accepted : 2018-11-06

ABSTRACT

In-stent restenosis after stenting in vascular occurs secondary to the accumulation of smooth muscle cell and extracellular matrix. This condition is a major complication caused by the occurrence of neointimal hyperplasia (NIH). The study aimed to prove the role of miRNA-21 as a risk predictor of the NIH event in aorta of rabbits animal model who underwent bare metal (BM) type stent implantation and to know the miRNA-21 role in IL-6 and IL-8 expressions. This study was quasi experimental, coducted in Catheterization Laboratory Dr. Sardjito General Hospital Yogyakarta. Stent implantation was observed intravascular ultrasonography. Blood vessel which was already deployed by stent taken as sample for pathologic examination. Rabbit blood samples were collected on day 0, 7, and 28. Quantification miRNA-21 expression has been done with qPCR and ELISA approach. These 10 rabbits divided into two groups in which one group as control (without stent deployment, 4 models) and another group as intervention (with BM type stent deployment, 6 models). The increase in expression of miRNA-21 on the day 7 and day 28 in the intervention group compared to the control group. Neointimal hyperplasia increased in intervention group on day 7, and 28 were from mild grade to moderate, and severe respectively. In addition, levels of IL-6 and IL-8 increased on day 28 compared with on day 7. This study showed increase of miRNA-21 expression on day 7 and NIH rise from the mild to moderate. Furthermore, on day 28, that increases of miRNA-21 expression and NIH rose from to moderate to severe. The increase of miRNA-21 was also shown on day 7 and 28 followed by the increase of IL-6 and IL-8 levels.

ABSTRAK

In-sten restenosis setelah proses pemasangan sten pada vaskular terjadi akibat akumulasi sel otot polos dan matriks ekstraselular. Kondisi ini merupakan komplikasi utama yang disebabkan oleh terjadinya neointimal hyperplasia (NIH). Penelitian ini bertujuan untuk membuktikan peran miRNA-21 sebagai prediktor risiko NIH pada aotrta dari model hewan kelinci yang diperlakukan implantasi stent tipe bare metal (BM) dan miRNA-21 pada ekspresi IL-6 dan IL-8. Penelitian ini merupakan experimen quasi yang dilaksanakan di Laboratorium Catheterisasi, RSUP Dr. Sardjito Yogyakarta. Implantasi sten diamati dengan intravascular ultrasonografi. Pembuluh darah yang telah dipasangi dengan stent diambil sebagai sampel untuk pemeriksaan patologi. Darah kelinci dikumpulkan pada hari ke 7 dan 28. Kuantifikasi ekspresi miRNA-21 dilakukan dengan metode qPCR dan ELISA. Kelinci sebanyak 10 ekor dibagi menjadi dua kelompok. Kelompok kontrol sebanyak 4 ekor tanpa pemasangan stent. Kelompok perlakuan sebanyak 6 ekor dengan pemasangan stent tipe BM. Ekspresi miRNA-21 meningkat pada hari ke 7 dan 28 pada kelompok perlakuan dibandingkan kontrol. Peningkatan NIH pada kedua kelompok pengamatan hari ke 7 dan 28 tergolong ringan, sedang hingga berat. Kdar IL-6 dan IL-8 juga mengalami kenaikan. Ekspresi miRNA-21 meningkat hari ke 7 dan NIH meningkat dari ringan hingga sedang. Pada hari ke 28 terjadi peningkatan ekspresi miRNA-21 dan NIH dari sedang menjadi berat. Penigkatan miRNA-21 juga terlihat pada hari ke 7 dan 28 dengan disertai kenaikan kadar IL-6 dan IL-8.

Keywords:
miRNA-21
bare metal stent
interleukin-6
interleukin-8
neointimal hyperplasia

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INTRODUCTION

Cardiovascular diseases which have high morbidity and mortality are considered having high global burden every year. According to WHO, in 2015 it was estimated deaths from cardiovascular diseases would be increased to 20 million. Nearly one third of the incidence of global deaths is due to cardiovascular diseases.[1] Based on WHO (2011), death from coronary heart disease (CHD) reached 234,000 or 17.05% of total death in Indonesia. The age-adjusted death rate is 150.77 per 100,000 population which ranks Indonesia 51 in the world.[2]

Combination of lifestyle modification, medical procedures and invasive procedure to open or dilate the narrowed or blocked coronary arteries are the therapeutic choices of CHD. Percutaneous intervention in which considered as invasive procedures including angioplasty using balloons and other invasive procedure like stent placement in the coronary arteries. Percutaneous coronary intervention (PCI) is a therapeutic revolution that develops rapidly and very important in CHD patients where previous coronary blood flow is lacking or even completely blocked so that the vessel becomes open.[3]

Some issues are still open and represent a chalenge despite continuous technical advances in stent restenosis (ISR). This condition is a major complication caused by the occurrence of neointimal hyperplasia (NIH).[3] The pathophysiological mechanism of restenosis is still not well explained, but it is believed that various mechanisms involved including inflammation, proliferation and remodeling of the matrix. In recent years, several clinical, biological, genetic, epigenetic predictors have been identified related to lesions and procedural risk factors for restenosis.[4]

Hence, early detection and prevention of restenosis to produce better clinical outcomes are needed. Reliable biomarker in which considered minimal invasive measurement is one of the modality. Currently, a micro-ribonucleic acid (miRNA) attracts the attention of researchers in the world due to its role in ISR. The miRNA is small RNA, with a length of 20-25 nucleotides, single stranded and have no role in encoding proteins, yet play a role in regulating target gene expression at the post transcription level. It further reduces protein expression by blocking the translation of mRNA and by promoting proteins degradation.[5]

miRNA has the ability to regulate systemic functions such as inflammatory responses.[6] This role is prominent in the initiation and resolution of inflammation after the onset of vessel injury.[7] miRNA is very important because its involvement in various biological processes in NIH after the installation of coronary stents. miRNA also detected in the blood circulation and may be useful as a biomarker for cardiovascular diseases.[8]

Various miRNAs have the function of downregulation the inflammatory pathways, which are biologically useful for controlling the inflammatory process by targeting specific proteins.[9] Modulation over miRNA-21 expression *in vitro*, with depletion has a negative effect from neointimal lesion formation. *In vitro* expression of miRNA-21 levels shows significantly higher in vascular smooth muscle cells differentiated than non-differentiated cells. Depletion or decrease of miRNA-21 causes a decrease in proliferation and increase apoptosis of the cells. The cellular effect of miRNA-21 was shown by *in vivo* studies of carotid arteries of rats that had lesions induced by balloon intervention. Moreover, analysis with westernblot shows that PTEN and Bcl-2 are the target proteins of miRNA-21 cellular effects. These results indicate that miRNA-21 regulates and stimulates the formation of neointimal lesions.[10]

MiRNA-21 plays an important role in vascular inflammation promotion and lesion remodeling after stent insertion in pig animal model.[7] Anti miRNA-21 inhibits the proliferation of vascular smooth muscle cells both *in vitro* and *in vivo*.[3] Depend on the presence of a stimulus, vascular smooth muscle cells are able to turn into a proliferative or

differentiate state. This study aimed to prove the role of miRNA-21 as a risk predictor of the NIH event in aorta rabbits animal model who underwent BM type stent implantation and to evaluate the miRNA-21 role in IL-6 and IL-8 expressions. It is expected that miRNA-21 can be used as an early predictor of the occurrence of neo-intimal hyperplasia after BM stent installation.

MATERIALS AND METHODS

Animal model

Design of this study is quasy.We used male *Oryctolagus cuniculus* rabbits, 4 rabbits as control group and 6 rabbits as treatment group. Study had been done in Catheterization Laboratory Dr. Sardjito General Hospital, Yogyakarta. Stent implantation and intravascular ultrasonography performed to observe and ensure the position and stent deployment were optimized.

Protocol of study

Blood vessel which was already deployed by stent was taken as sample for pathologic examination. Thickening of the NIH measured by accounting the number of smooth muscle cells that arranged in the inner lines of the lumen of the artery by microscop observations on cross sectional vascular preparation. All sprocedures were performed by certified operators with standard operation procedures. Protocol of the study has been approved by the Research Ethics Committee for Animal Laboratory of the Integrated and Testing Laboratory (*Laboratorium Penelitian dan Pengujian Terpadu*/LPPT), Universitas Gadjah Mada Yogyakarta number 00030/04/LPPT/VI/2016.

Blood samples were collected from the rabbit ear on day0, day 7, and day 28. There is no predetermine time collection since there is still not any references taken into account the best time to collect miRNA in blood sample. Quantification expression had done in several steps with qPCR and ELISA approach. Quantification of miRNA was calculeted with qPCR and quantification of IL using ELISA. The primers for miRNA-24 were CGCCTGGCTCAGTTCAGCA (forward) GCAGCTCTTCATTTACGGTCCA (reverse), for miRNA-2 was GCACCGTCAAGGCTGAGAAC (forward) and CAGCCCATCGACTGGTG (reverse) with 40 cycles. Smooth muscle cell quantified using smooth muscle cell-actin staining. ELISA kits FineTestwere used for IL quantification catalogue number ERB0068 for IL-6 and catalogue number ERB0069 for IL-8.

Statistical analysis

Data were presented as mean ± standard error of the mean (SEM) and analysed using SPSS version 23. A p value < 0.05 was considered significance.

RESULTS

Ten O. cuniculus male rabbits was used in this study. The rabbits divided into two groups i.e. control grou without stent deployment and treatment group with BM type stent deployment. The characteristics the animal models are presented in TABLE 1. No any abnormality and differences between two groups in terms of nutrition and hematology profile were observed. Medication were different between those two groups as consequence of stent deployment. Aspirin and clopidogrel have been administered since 7 days before and after 28 days after intervension. After the trial was done, all rabbits were euthanased according to standard procedure.

TABLE 1. Characteristics of animal models

Parameters	Control group (n=4)	Treatment group (n=6)
Rabbit type	*O. cuniculus*	*O. cuniculus*
Age (mean ± SEM months)	8.1± 0.13	8.2±0.15
Male sex (%)	100	100
Weight (mean ± SEM kg)	3.9 ±0.43	3.9±0.48
Nutrition status	Fair	Fair
Hematology profile		
• Hemoglobin	10.8±0.86	11.2±2.47
• Leukocyte count	6.9±2.70	7.8±5.4
• Ureum	20.7±7.43	16.0±2.72
• Creatinine	0.8±1.15	1.90±0.41
Medication		
• Placebo	Yes	No
• Aspirin (mg/kg/day)	No	1
• Clopidogrel (mg/kg/day)	No	1

Cycle quantification was done in miRNA-21 (TABLE 2) which shown that there were no differences between day 0, 7, and 28 in control group (p>0.05). The miRNA-21 expression were significantly increase after intervention with BM type stent compared with control group on day7 (5.77 ± 0.67 versus 1.50 ± 0.59, p=0.001; 95%CI 2.830-5.697) and day 28 (7.31 ± 0.41 versus 1.73 ± 0.44; p =0.009; 95%CI 4.605-6.554). The increase of the miRNA-21 after intervention with BM type stent was also observed on day28 (7.31 ± 0.41) compared with on day7 (5.77 ± 0.67) as shown on TABLE 3 and FIGURE 1. However, it was not significntly different (p = 0.092).

TABLE 2. Expression quantification result by Cq (cycle quantification) miRNA in control group

Days	miRNA-21 ± SEM
0	35.68 ± 0.13
7	35.72 ± 0.16
28	33.79 0.58
p	0.87[a]; 0.37[b]; 0.26[c]

[a]Control on day0 versus intervention in day 7; [b]Control on day0 versus intervention in day 28; [c]Control on day7 versus intervention in day 28

TABLE 3. Differences of miRNA-21 expression between intervention and control group on day7, and day 28.

Days	Control group	Intervention group	p (95%CI)
7	1.50 ± 0.59	5.77 ± 0.67	0.001 (2.830-5.697)
28	1.73 ± 0.44	7.31 ± 0.41	0.009 (4.605-6.554)
p	0.260	0.092	

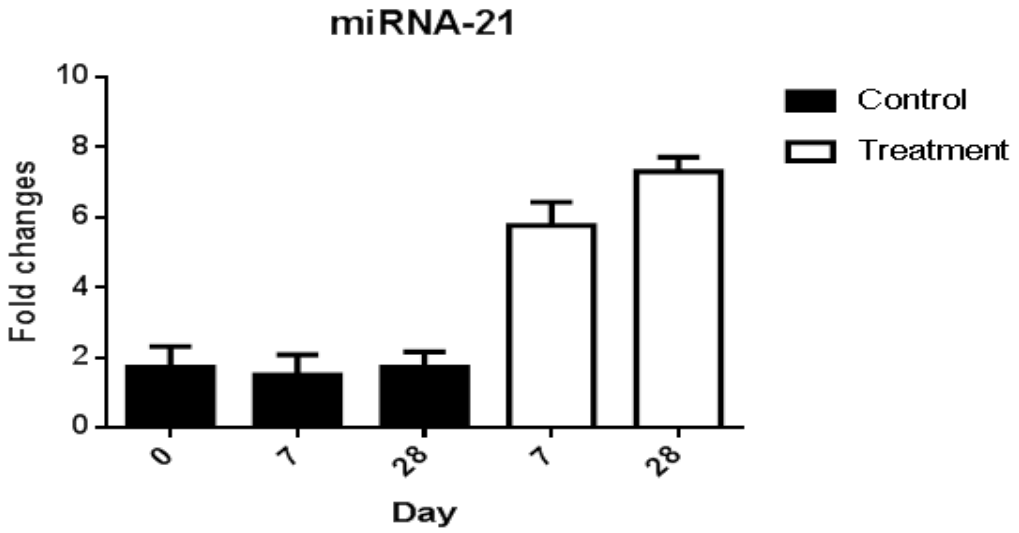

FIGURE 1. Level of miRNA-21 expression (fold change) on day0, 7, and 28 between control and intervention group.

Vascular smooth muscle cells number also measured in control group with the lowest number was 8 and the highest was 13. Intervention with BM type stent (intervention group) showed 10 as the lowest number while 35 as the highest number (TABLE 4).

TABLE 4. Number of vascular smooth muscle cells both in control and intervention groups

Observation	Mean ± SD	p
Days 0		
• Control	11.00 ± 0.00	
• Intervention	-	
Day 7		
• Control	10.00 ± 0.00	
• Intervention	14.88 ± 3.15	0.397[a]; 0.311[b]
Days 28		
• Control	10.16 ± 1.64	
• Intervention	28.19 ± 4.72	0.079[c]; 0.0.015[d]; <0.016[e]; 0.016[f]

[a]Control group on day0 versus intervention on day7; [b]Control group on day7 versus intervention on day7; [c]Control group on day7 versus intervention on day28; [d]Intervention group on day7 versus intervention on day28; [e]Comparison control group day 28 with intervention group day 28.

Three categories are divided based on these numbers by tertiel percentage (TABLE 5) to show the severity of NIH.

TABLE 5. Three divided categories between vascular smooth muscle cells

Severity of neo intima hyperplasia	Vascular smooth muscle cells
Mild	8 - 11
Moderate	12 - 16
Severe	17 - 35

The mean of NIH in control group from day 0, intervention group day 7, and intervention group day 28 was 10.38 ± 0.37 (mild), 14.88 ± 1.04 (moderate), and 28.19 ± 2.37 (severe) respectively. Significantly increase of NIH between intervention group on day28 compared with on day7 was observed (p = 0.004; 95% CI -3.94 – 17.68).

The level of IL-6 between control and intervention group on day0, 7 and 28 are shown on TABLE 6 or FIGURE 2. In general, there was no significantly different of the level of IL-6 between control group on day0, 7 and 28 compared with intervention group on day0, 7 and 28 (p>0.05), except the level of IL-6 between control group on day0 compared with intervention group on day28 (p = 0.005).

TABLE 6. IL-6 level (mean ± SEM ng/L) 0, 7, and 28 both in control and intervention group

Group	n	IL-6	p
Control group day 0	3	0.08 ± 0.02	
Control group day 7	1	0.04	0.19[a]
Control group day 28	1	0.09	0.005[b]
Intervention group day 7	3	0.32 ± 0.15	0.18[c]
Intervention group day 28	3	0.50 ± 0.07	0.18[d]; 0.51[e]

SEM = standart error of the mean; [a]Comparison control group day 0 and intervention group day 7; [b]Comparison control group day 0 and intervention group day 28; [c]Comparison control group day 7 and intervention group day 7; [d]Comparison control group day 28 and intervention group day 28; [e]Comparison intervention group day 7 and intervention group day 28

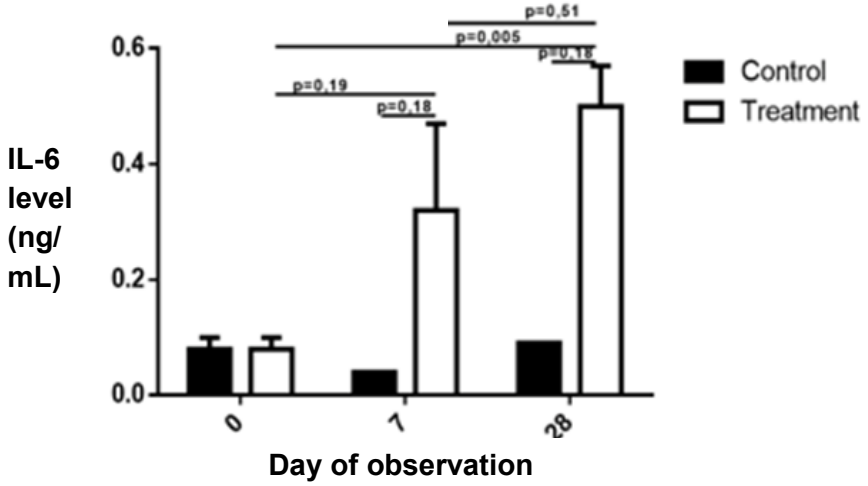

FIGURE 2. Level of IL-6 in days 0,7, and 28 between control and intervention group

The level of IL-8 between control and intervention group on day0, 7 and 28 are shown on TABLE 7 or FIGURE 3. Significantly different in the level of IL-8 between control group on day0 and intervention group on day7 (p=0.001), between control group on day0 and intervention group on day28 (p<0.001) as well as intervention group on day7 and intervention group on day18 (p<0.05) were observed.

TABLE 7. IL-8 level (mean ± SEM ng/mL) on day0, 7, and 28 both in control and intervention group

Group	n	IL-8	p
Control group day 0	3	0.37 ± 0.10	
Control group day 7	1	0.49	
Control group day 28	1	0.32	
Intervention group day 7	3	1.47 ± 0.06	0.001[a]; 0.18[b]
Intervention group day 28	3	2.24 ± 0.04	<0.001[c]; <0.05[d]; 0.18[e]

SEM = standard error of the mean; [a]Comparison control group day 0 and intervention group day 7; [b]Comparison control group day 7 and intervention group day 7; [c]Comparison control group day 0 and intervention group day 28; [d]Comparison intervention group day 7 and intervention group day 28; [e]Comparison control group day 28 and intervention group day 28.

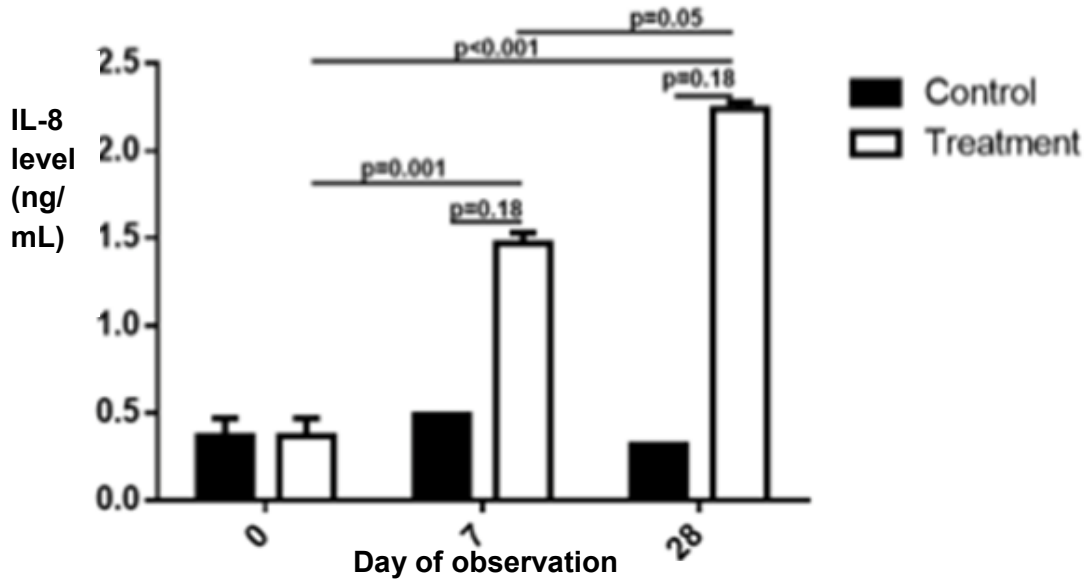

FIGURE 3. Level of IL-8 in day 0, 7, and 28 between control and intervention group

DISCUSSION

miRNA-21 expression after intervention with BM type stent significantly increased 5.77 times on day 7 (p = 0.001; 95% CI: 2.830-5.697) and 7.31 times on day 28 (p = 0.00117; 95% CI: 2.830-5.697) compared with control. Although, there was an increase 1.26 times of miRNA-21 expression after intervention with BM type stent on day 28 compared with that on day7, however it was not significantly different (p=0.092).

Gibbons and Dzau[12] found that the miRNA-21 expression increased after vascular injury lead proliferative effect on vascular smooth muscle cells. Neointimal hyperplasia is characterized by the proliferation and migration of vascular smooth muscle cells. It is also shown by increasing in the extracellular matrix, which can lead in narrowing and blockage of the blood vessels lumen.[3] Then, miRNA-21 expression increases in tissues of human patients who experience in stent restenosis compared with specimens of coronary heart disease patients who are not deployed with stents.[3] A study conucted by Halliday[13] showed the important role of miRNA-21 in the occurrence of in stent restenosis. Moreover, by removing the gene from miRNA-21, it is shown to reduce the incidence of in stent restenosis through the proliferation and migration response of vascular smooth muscle cells and also change the immunity function of the cell.

miRNA-21 through its suppression function has practical implications in increasing cell proliferation, inflammation, cell replication, abnormal metabolism, angiogenesis, apoptosis avoidance, immune destruction and suppressor growth. Specifically miRNA-21 strongly influences the apoptosis process.[14] miRNA-21 plays a large role in vascular inflammation stimulation and post-lesion remodeling due to the deployment of stents in pigs.[7] Furthermore, anti miRNA-21 inhibits the proliferation of vascular smooth muscle cells both *in vitro* and *in vivo*.[3] miRNA-21 has a molecular target of PTEN, BMPR2, WWP1, YOD1, and SATB1 which have the function as proliferation inhibition of vascular smooth muscle cells. miRNA-21 suppresses the target so that the proliferation process goes continuously without any inhibition.[15] miRNA-21 also has an important influence in increasing IL-6. Expression of miRNA-21 increased in inflammatory tissue with increased IL-6 levels compared to control tissue, so it was concluded that miRNA-21 has a role in the inflammatory process.[16] This study also carried out in patients with intervertebral disc degeneration.

In patient with intervertebral disc degeneration, there was an increase of cytokine IL-6 (125.6 $\pm$ 34.9 umol/L) with increase of miRNA-21 expression compared to healthy group ($p < 0.001$).[16] Another study proved that miRNA-21 is strongly associated with inflammation. A strong association between miRNA-21 expression and inflammatory markers of IL-6 and IL-8 suggests that there was a positive relationship between miRNA-21 function and inflammatory effects.[11] Following percutaneous coronary intervention (PCI), short-term clopidogrel therapy in addition to aspirin leads to greater protection from thrombotic complications than aspirin alone. Clopidogrel and aspirin treatment before stenting is used for protection of thrombotic complications. The antiplatelet effects of these drugs persists for around one week, which corresponds to the mature platelet lifespan.[17]

This study has some limitations because we cannot observe all the miRNA in which may associate with IL-6 and IL-8 measurement. We also id not perform randomization in rabbits animal model since it is not feasible in this study.

CONCLUSION

This study showed there is an

increase of miRNA-21 expression on day 7 and neo-intimal hyperplasia rise from the mild to moderate after intervention with BM type stent. Furthermore, on day 28, there is an increase of miRNA-21 expression and neo-intimal hyperplasia rise from to moderate to severe. The expression of miRNA-21 increased after vascular injury lead proliferative effect on vascular smooth muscle cells. The increase of miRNA-21 is also shown on day 7 and day 28 with increase of IL-6 and IL-8 value. It can be concluded that miRNA-21 might have a role in the inflammatory process.

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