HOSTED BY
ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage: http://ees.elsevier.com/apjtm

Original research http://dx.doi.org/10.1016/j.apjtm.2015.09.015

Blood pH in coronary artery microthrombosis of rats

Kuai-Fa Fang[1,3,4,#], Zhu-Jun Chen[2], Meng Liu[1], Ping-Sheng Wu[1*], Da-Zhi Yu[5,6,#]

[1]*Department of Cardiology, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong Province 510515, China*

[2]*Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangzhou, Guangdong Province 510080, China*

[3]*Department of Cardiology, Affiliated Huiyang Hospital of Southern Medical University, Huizhou, Guangdong Province 516200, China*

[4]*Department of Cardiology, Huizhou No.6 People's Hospital, Huizhou, Guangdong Province 516200, China*

[5]*Postdoctoral Research Station, Ji'nan Military General Hospital, Ji'nan 250000, China*

[6]*The PLA 401 Hospital, Qingdao 266071, China*

ARTICLE INFO

Article history:
Received 15 Jul 2015
Received in revised form 20 Aug 2015
Accepted 15 Sep 2015
Available online 25 Sep 2015

Keywords:
pH
Coronary artery
Microthrombus
FGL2

ABSTRACT

Objective: To study the mechanism and significance of pH change in the coronary artery microthrombosis of rats.

Methods: After the sodium laurate-induced model of coronary artery microthrombosis of rats was constructed, the vascular endothelial cells were separated and then cultured in the mediums with different pH values for 24 h. Enzyme linked immunosorbent assay was used to detect the content of von Willebrand factor (vWF) in the medium; while the real-time PCR and western blot assay were used to detect the expression of fibrinogen-like protein 2 (FGL2) at the mRNA and protein level. The comprehensive evaluation was performed to discuss the effect of pH change on the coronary artery microthrombosis of rats.

Results: The expression level of vWF detected by enzyme linked immunosorbent assay was 336.67 ± 24.95, 311.33 ± 14.98, 359.67 ± 39.63, 354.67 ± 49.01 and 332.00 ± 33.42 (pg/mL) respectively; while the expression of vWF in the model group was 570.00 ± 57.94, 524.67 ± 57.94, 437.00 ± 95.38, 415.33 ± 44.38 and 444.67 ± 74.31 respectively. Being cultured under the different pH values, the relative expression level of *FGL2* mRNA in the model group was 7.93 ± 0.93, 6.70 ± 0.70, 5.03 ± 0.32, 5.13 ± 0.40 and 5.57 ± 0.83 respectively.

Conclusions: The coronary artery microthrombosis of rats can cause the high expression and secretion of vWF. Meanwhile, *FGL2* is also up-regulated in the thrombosis and such up-regulation is more significant in the condition with low pH, which indicates that the low pH condition may be one of factors that contribute to the cardiovascular diseases.

1. Introduction

The cardiovascular diseases have become the major public health problem and the microthrombus-induced microcirculatory disturbance of coronary artery has been increasingly emphasized in the treatment of cardiovascular diseases. According to previous researches, the normal blood pH was a key factor to maintain the cardiovascular homeostasis [1] and the change in blood pH could destroy the homeostasis of circulatory system and thus cause a series of cardiovascular diseases [1,2]. Therefore, it's of critical importance to study the mechanism of pH in changing the coronary artery microthrombosis [3].

In this study, relying on the construction of the coronary artery microthrombosis model of rats, the microvascular endothelial cells were separated and then cultured in the medium with the different pH (pH 6.4–pH 8.6). Enzyme linked immunosorbent assay (ELISA) was used to detect the content of vWF in the

*Corresponding author: Ping-Sheng Wu, Ph.D, attending physician, Department of Cardiology, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong Province 510515, China.
Tel: +86 13640739361
E-mail: kuaifa@gmail.com

Peer review under responsibility of Hainan Medical College.
Foundation project: It is supported by Medical Scientific Research Foundation of Guangdong Province (No. A2012242).

# These two authors are co-first authors.

1995-7645/Copyright © 2015 Hainan Medical College. Production and hosting by Elsevier (Singapore) Pte Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

medium, as well as the expression of fibrinogen-like protein 2 (FGL2) at the mRNA and protein level, in order to study the mechanism and significance of pH change in the coronary artery microthrombosis of rats.

2. Materials and methods

2.1. Materials

2.1.1. Laboratory animals

20 specific pathogen free male or female SD rats with the weight of (250 ± 50) g were purchased from Better Biotechnology Co., Ltd. and they were randomly divided into the control group (5 rats) and experimental group (15 rats). All rats were fed the standard pellets in the standard animal cage, with 5 rats in each cage. Rats could eat and drink freely during the experiment. The feeding room had the good ventilation, with the natural lighting day and night. The room temperature was maintained at 18–25 °C.

2.1.2. Reagents and instruments

The sodium laurate was purchased from Sigma; DMEM medium from GIBIC; von Willebrand factor (vWF) and ELISA kit from Uscn Life Science Inc. Wuhan; pentobarbital sodium from Sigma; RNA extraction kit (RNeasy Plus Mini Kit) from QIAGEN; reverse transcription kit (iScript cDNA Synthesis Kit) from Bio-Rad; real-time fluorescent quantitative PCR kit (SsoAdvanced SYBR Green Super mix) from Bio-Rad; and FGL2 monoclonal antibody from Abcam.

CO_2 constant temperature incubator: SANYO; UV spectrophotometer: UV-1750, Shimadzu; pH meter: pH meter 345 Coring; fluorescent quantitative PCR system: CFX96 Touch, Bio-Rad.

2.2. Methods

2.2.1. Construction of animal model of coronary artery microthrombosis of rats

Rats in the model group of coronary artery microthrombosis (n = 15) were anaesthetized by pentobarbital sodium with the dosage of 45 mg/kg and they were fixed in the supine position. After the skin preparation and sterilization, the endotracheal intubation was performed and the small animal ventilator was adopted for the assisted ventilation. The horizontal cutting was done at the 2nd intercostals space to expose the heart and open the pericardium. 0.5 mm needle was placed in the root of aorta and then the clamp was used to hold the aorta. Rats in the model group were injected by 1.0 mg/kg sodium laurate (20 mg/mL), while rats in the control group were injected by 0.9% NaCl (n = 5). Then the clamp was loosened, with hemostasis by compression. It was then injected by 800 000 units penicillin, following by the sternal closure for the recovery [4].

2.2.2. Separation and culture of microvascular endothelial cells

At 1 h after the operation, rats were anaesthetized by the pentobarbital sodium. Hearts of rats were separated rapidly and they were washed by the cold normal saline once. Tissues with the vessel were cut off from the sinus ostium of aorta, namely the origin of coronary artery. Then the connective tissues were dissected from the surface and the coronary artery was separated. It was washed by the aseptic and pre-cooled normal saline for three times. Tissues were cut into 1 cm^3 blocks using the ophthalmic scissors and then they were transferred into the tissue culture flask. 0.25% trypsin (EDTA) was added. It was then put in the shaker for 4 h of enzymolysis at 4 °C. After the enzymolysis, the pipette was used to blow the digestive fluid to separate the cells [5–7].

The digested mixture of tissue blocks was filtered through the 20 μm filter mesh, in order to remove the undigested tissue blocks. The suspension was collected and then prepared for the vortex oscillation to centrifugally remove the trypsin. DMEM medium was used for the re-suspension. It was then cultured in the tissue culture flask ($HCl/NaHCO_3$ to regulate the medium pH). Afterwards, 20% fetal bovine serum was added and then it was cultured in the incubator with 5% CO_2 and at 37 °C.

2.2.3. Content of vWF in cell culture medium detected by ELISA

USCNK vWF ELISA kit (USCNK SEA833Ra) was employed to perform the quantitative detection of vWF in the cell culture medium, with the specific steps as follows:

The standard well, sample well and blank well were set respectively, where in 7 standard wells, the standard substance with different concentration was added once, as well as the samples. The ELISA plate was covered with film and it was incubated at 37 °C for 2 h. A total of 100 μL test solution was added in each well (biotinylated vWF antibody). The ELISA plate was covered with film and it was incubated at 37 °C for 1 h. The fluid in the well was removed and then each well was washed with 350 μL scrubbing solution. It was soaked for 1–2 min and then the plate was washed for 3 times. A total of 100 μL HRP-labeled secondary antibody was added in each well. The ELISA plate was covered with film and it was incubated at 37 °C for 30 min. A total of 90 μL TMB substrate was added in each well. The ELISA plate was covered with film and it was colored in a dark place at 37 °C for 15–25 min. When the first 3–4 standard wells appeared the obvious gradient blue, the reaction was stopped and then 50 μL 2M H_2SO_4 was added. OD value of each well was measured at 450 nm of enzyme-labeled instrument immediately.

All data were expressed by mean ± standard deviation (SD). The t test was performed on the comparison between two groups, while the one-way analysis of variance and LSD-t test were performed on the comparison among groups. $P < 0.05$ was treated as the statistical significant difference.

2.2.4. Real-time PCR

The cultured primary cells were collected. It was then washed with PBS for 3 times and centrifuged to remove the supernatant. 1 mL Trizol was used for the lysis of every 10^7–10^8 cells. The pipette was used to blow and mix the solution. It was then put at the room temperature of 15–30 °C for 5 min. 0.2 mL chloroform was added in each 1 mL Trizol. It was covered with Eppendorf cap and then oscillated in vortex for 15 s. It was put at the room temperature for 2–3 min and then centrifuged at 12 000 g and 4 °C for 10 min. The supernatant was collected and transferred

to the new Eppendorf tube. 0.5 mL isopropyl alcohol was added in each 1 mL Trizol. It was put at the room temperature for 10 min and then centrifuged at 12 000 *g* and 4 °C for 10 min. The supernatant was removed. 1 mL 75% ethanol (DEPC-treated water) was added in each 1 mL Trizol for the washing and vortex mixing. It was then centrifuged at 7 500 *g* and 4 °C for 5 min and the supernatant was removed. The precipitated RNA was naturally dried at the room temperature. RNA precipitation was dissolved with the Rnase-free water. The UV spectrophotometer was used to measure the concentration of RNA.

Real-time PCR was used to detect the Ct value of gene amplification, while Ct value was negatively correlated to the initial copy number of DNA. The relative quantitative method was employed, using *GAPDH* as the reference, to analyze ΔCt of each sample, where ΔCt = Ct of target gene − Ct of reference gene, and ΔCt referred to the value of data analysis. *FGL2* and *GAPDH* mRNA sequence were checked in the database of NCBI. Then the primers of Real-time PCR were designed and all primers were synthesized by Invitrogen. Specific sequences were shown in the following Tables 1–3.

Table 1

Primer used in Real-time PCR.

Gene	Accession no.	Primer (5′-3′)
FGL2	NM053455.2	For: ggaatgctgagggccatgtta
		Rev: aaacactgcgccctgtctga
GAPDH	NM002046	For: gatggtgaaggtcggtgtg
		Rev: gaggtcaatgaaggggtcg

GAPDH, glyceraldehyde phosphate dehydrogenase; *FGL2*, fibrinogen-like 2; For, forward; Rev, reverse.

Table 2

Synthetic system of inverse transcription.

Components	Volume per reaction
5 × iScript reaction mix	4 μL
iScript reverse transcriptase	1 μL
RNA template (1 μg RNA)	1 μg
Nuclease-free water	Up to 20 μL

iScript reaction mix was purchased from Bio-Rad and nuclease-free water from Invitrogen.

Table 3

Synthetic system of PCR.

Components	Volume per reaction
SsoAdvanced SYBR Green Super mix	5 μL
Forward primer (10 μM)	0.3 μL (300 nM)
Reverse primer (10 μM)	0.3 μL (300 nM)
cDNA template	100 ng
Nuclease-free water	Up to 10 μL

SsoAdvanced SYBR Green Super mix was purchased from Bio-Rad.

2.2.5. Western-blotting to detect the expression of FGL2

The collected cells were lysed with 50 μL RIPA lysis buffer. Then the protease inhibitor cocktail was added [RIPA/cocktail = 100/1(V/V), namely 10 μL cocktail in 1 mL RIPA by volume]. It was blew and mixed. After being put on the ice for 30–40 min, cells were lysed using the ultrasound. The probe-type ultrasound was used to produce the short impact with the appropriate frequency on the ice. The lysis mixture was centrifuged at 4 °C and 13 000 r/min for 20 min. The supernatant was transferred to the new centrifuge tube. Protein Assay Kit was employed to detect the protein concentration.

SDS-PAGE electrophoresis. The gel was soaked in the transfer buffer for 10 min of equilibrium. It was installed with the transfer ‘sandwich’ and the transfer buffer was added. The electrodes were inserted, with 100 V and for 45–60 min. After the transfer, PVDF film was washed with TBS for 10–15 min. The film was placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder and shaken at the room temperature for 1 h. The film was washed with TBST for 3 times and 5–10 min each time. Then the primary antibody with the appropriate degree of dilution was added [diluted with TBST containing 1% (w/v) skimmed milk powder]. It was incubated at the room temperature for 2 h and then the film was washed with TBST for 3 times and 5–10 min each time. The film was incubated with the secondary antibody (1:10 000, HRP-labeled) that was diluted with TBST containing 0.05% (w/v) skimmed milk powder. It was incubated at the room temperature for 1 h and then the film was washed with TBST for 3 times and 5–10 min each time. It was exposed and then photographed to save the experimental results. The experiment was repeated three times. Quantity one v4.62 was used to measure the gray value of molecular band (trace tracking method of band). The optical density curve was drawn according to the optical density of different electrophoretic band. The area under the optical density curve was calculated as the quantitative basis for the electrophoretic bands and the statistical analysis was performed on collected data.

2.3. Statistical analysis

The experimental data was treated using SPSS 12.0. Results were expressed by mean ± SD. The analysis of variance, student-*t* test and one-way ANOVA were performed, where $P < 0.05$ referred to the statistically significant difference.

3. Results

3.1. Detection of Vwf content in tcell culture medium

The separated microvascular endothelial cells were cultured for 12 h in the mediums with different pH and then ELISA was used to detect the vWF content in the supernatant of medium.

As shown in Table 4, there was little change in the expression of vWF in the supernatant of microvascular endothelial cells that were cultured under the different pH values (pH 6.4–pH 8.0) in the control group. But in the model group, as the thrombosis model was induced with the sodium laurate and a great number of vWF was expressed and secreted, it could be indicated that the expression of vWF under the low pH was higher than ones of treatment group under pH 7.2–8.0, but the difference was not significant.

Table 4
Expression of vWF (pg/mL, mean ± SD).

Group	pH				
	6.4	6.8	7.2	7.4	8.0
Control	336.67 ± 24.95	311.33 ± 14.98	359.67 ± 39.63	354.67 ± 49.01	332.00 ± 33.42
Model	570.00 ± 57.94	524.67 ± 57.94	437.00 ± 95.38	415.33 ± 44.38	444.67 ± 74.31

3.2. Expression of FGL2 gene in cultured microvascular endothelial cells

For vascular endothelial cells cultured in the medium with different pH values for 12 h, as shown in Figure 1, there was no significant change in the expression of *FGL2* in the control group under the different pH values ($P > 0.05$); while in the model group, the relative expression of *FGL2* mRNA under the low pH value in the model group was higher than the one under the high pH value. The relative expression of *FGL2* mRNA cultured under the different pH values was shown in Table 5 (the relative expression was automatically obtained by the software, ΔCt = Ct of target gene − Ct of reference gene, ΔCt as the value of data analysis). It could be seen that, under the low pH, the relative expression of *FGL2* mRNA was significantly higher than the one of the treatment group under the pH 7.2–8.0 ($P < 0.05$), which was positively correlated with the change in the content of vWF in the medium supernatant.

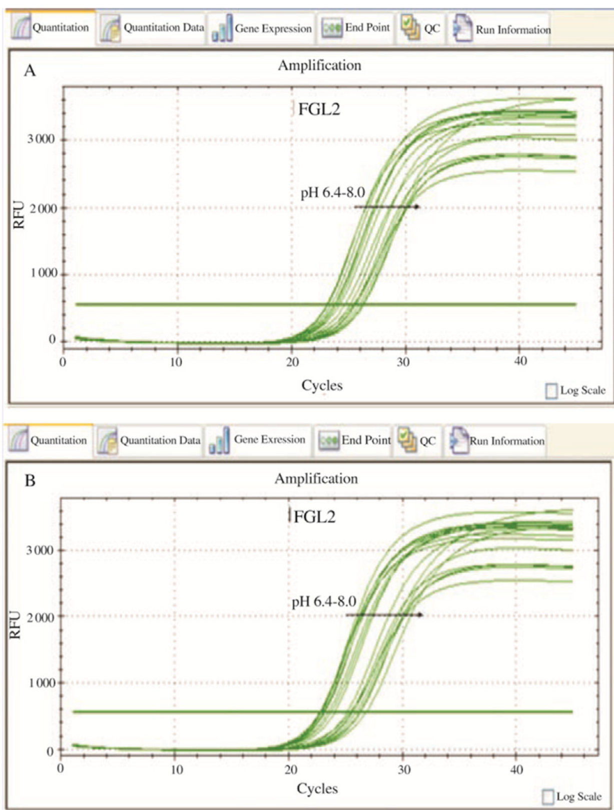

Figure 1. Normalized mRNA expression. A: control group; B: model group.

3.3. Expression of FGL2 protein in cultured microvascular endothelial cells

The western-blotting was used to detect the expression of FGL2 protein in the microvascular endothelial cells that were cultured in the medium under the different pH values. According to Figure 2, in the thrombosis model group, the expression of FGL2 that was cultured under pH 6.4 and pH 6.8 was significantly higher than ones of other groups. There was little change in the expression of FGL2 in treatments of model group under other pH values. In the control group, there was little change in the expression of FGL2 under different pH values (the expression in the group under pH 8.0 was a bit down-regulated). As a whole, the expression of FGL2 in the control group was significantly lower than ones in the model group. Table 6 showed the results of gray values of bands that were analyzed by Quantity One v4.62. According to the results, under the pH 6.4 and pH 6.8, the band signal of FGL2 was stronger than ones under other pH values ($P < 0.01$); but there was no significant different between bands in the control group.

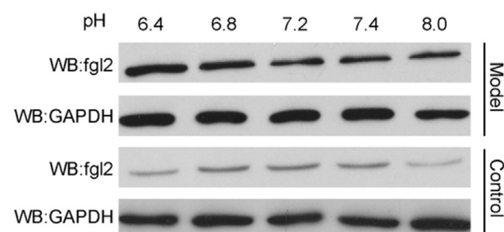

Figure 2. Expression of FGL2 in endothelial cells.

Table 5
Relative expression of *FGL2* in cultured microvascular endothelial cells.

Group	pH				
	6.4	6.8	7.2	7.4	8.0
Control	1.07 ± 0.32	0.97 ± 0.38	0.91 ± 0.17	0.98 ± 0.12	1.17 ± 0.42
Model	7.93 ± 0.93	6.7 ± 0.7	5.03 ± 0.32	5.13 ± 0.40	5.57 ± 0.83

Table 6

Statistics of band signal analyzed by quantity one.

Group	pH				
	6.4	6.8	7.2	7.4	8.0
Control	74.7 ± 5.3**	58.2 ± 2.4*	29.7 ± 12.1	30.2 ± 7.6	28.6 ± 3.3
Model	10.7 ± 1.7	15.7 ± 5.8	16.9 ± 1.9	15.7 ± 8.7	8.4 ± 9.0

*$P < 0.05$, **$P < 0.01$.

4. Discussion

The microcirculatory disturbance of coronary artery can cause the severe cardiovascular diseases such as the myocardial ischemia and myocardial infarction. The *in situ* microthrombosis is regarded as the major cause for the microcirculatory disturbance of coronary artery. However, the thrombosis is a complicated process involving multiple factors. The construction of coronary artery microthrombosis model of rats using the injection of sodium laurate in the aorta provided the favorable platform for the study of microthrombosis and the development of relative techniques [8,9].

The normal pH condition is essential for the organism to perform the normal metabolism. The pH of arterial blood should be maintained at 7.35–7.45. The pH disturbance is a key cause to cause the cardiovascular diseases. Therefore, the study of the role of pH in the thrombosis will contribute to exploring the occurrence and development of many cardiovascular diseases and providing the thoughts for the development of new cardiovascular drugs. In this study, relying on the construction of coronary artery microthrombosis model of rats, the microvascular endothelial cells were separated and then the medium pH was regulated to stimulate the microenvironment under the different pH values. Besides, using the important markers of thrombosis, it discussed the mechanism and significance of pH change on the coronary artery microthrombosis of rats, with the certain practical value [10,11].

There are many molecular markers to reflect the damaged vascular endothelium. By referring to many research findings, including vWF, Weibel–Palade, Thrombomodulin, Endothelin-1, Thromboglobulin and FGL2, it's found that FGL2, which was a related protein of fibrinogen found in recent years, could catalyze the prothrombin and thus form the active thrombin and quickly start the blood coagulation [12–14]. It was regarded that FGL2 played a key role in the microthrombosis [15–18]. Meanwhile, as vascular endothelial cells were involved in the physiological and pathological processes of development, regeneration and healing; in this study, FGL2 was chosen as a marker and to study its expression under different pH values in the vascular endothelial cells of rats in the coronary artery microthrombosis model, as well as the mechanism of pH change on the coronary artery microthrombosis of rats.

During the construction of coronary artery microthrombosis model of rats, it was found that there was abundant expression of vWF only 1 h after the operation. According to the pathological picture, the thrombosis had been formed. Thus vascular endothelial cells were separated and cultured 1 h after the operation [19]. The simple method of enzymolysis and filtering was used in the separation and culture of vascular endothelial cells. Though such method had the simple steps and it's easy to obtain much more cells and nearly impossible to cause the bacterium contamination, the obtained purity of cells was not high and it was easily contaminated by fibroblasts. If requiring the cells with the high purity, methods of magnetic beads sorting and density sedimentation should be employed for the further separation of cells [20], which should also be noticed and improved in the further study [7].

According to results of ELISA and compared with the control group, the expression of vWF in the endothelial cells of rats in the model group was significantly higher, which was also a marker of thrombosis. After the further culture and separation of vascular endothelial cells in the medium under different pH values, it could be seen that, in the medium under the low pH, the expression of vWF was significantly higher than ones under pH 7.2–8.0, which indicated that, in the low pH environment, it can change the activity of potassium channel and the tension of vessel wall and thus deeply impact the physiological function of the vessel [21]. Real-time PCR was used to detect the expression of *FGL2* at the mRNA level in cells. According to the similar findings, namely in the acidic condition, *FGL2* showed the high expression. Just like vWF, *FGL2* is also the marker of thrombosis. All these findings showed that the low pH condition will contribute to the thrombosis.

German scholar Rudolf Virchow proposed three factors of thrombosis in 1856, namely the vessel wall injury, stasis and hypercoagulability. Such theory was regarded as the foundation of thrombus researches. However, with the further studies on the thrombus, it is found that the thrombosis is not caused by a simple factor, but it is a complicated biological process involving multiple factors and at multiple levels, as the result of many factors. According to the experimental results in this study, it is regarded that the normal blood pH is a key factor to maintain the normal physiological state of cardiovascular. The pH disturbance will cause the change in the blood oxygen capacity and ion concentration, especially blocking the Cl^- channel, changing the exchange of Na^+/H^+ and Na^+/Ca^{2+} and the normal biological effects of Ca^{2+} channel [22,23]. Meanwhile, the low pH can also cause the generation of NO and the change in the resting tension of coronary vessels [3]. All these stimulus signals can activate the vascular endothelial cells to repair the related signal pathways, start the repair of damaged vessel wall, express the related fibrin and thrombin and thus break through the balance of procoagulant activity and anticoagulant activity and cause the thrombus finally. The effect of pH change on the coronary artery was observed in this study, while the molecular mechanism of microthrombosis will be deeply studied in the further experiments.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Brenninkmeijer L, Kuehl C, Geldart AM, Arons E, Christou H. Heme oxygenase-1 does not mediate the effects of extracellular

acidosis on vascular smooth muscle cell proliferation, migration, and susceptibility to apoptosis. *J Vasc Res* 2011; **48**(4): 285-296.

[2] Aoyama Y, Ueda K, Setogawa A, Kawai Y. Effects of pH on contraction and Ca^{2+} mobilization in vascular smooth muscles of the rabbit basilar artery. *Jpn J Physiol* 1999; **49**(1): 55-62.

[3] Lv Z, Zhang M, Zhang X, Liu Y, Fan J, Wu D, et al. Effect and mechanism of pH change on resting tension of *in vitro* coronary artery of rats. *Chin J Integr Med Cardio/Cereb Dis* 2011; **9**(12): 1474-1476.

[4] Ye M, Chen L, Chen D, Hong H. Coronary artery microthrombosis model of rats. *J Fujian Med Univ* 2003; **37**(1): 58-60.

[5] Hong F, Li Y, Xu Y. Decreased placental miR-126 expression and vascular endothelial growth factor levels in patients with pre-eclampsia. *J Int Med Res* 2014; **42**(6): 1243-1251.

[6] Plaisance-Bonstaff K, Choi HS, Beals T, Krueger BJ, Boss IW, Gay LA, et al. KSHV miRNAs decrease expression of lytic genes in latently infected PEL and endothelial cells by targeting host transcription factors. *Viruses* 2014; **6**(10): 4005-4023.

[7] Liang G, Jin H. Separation and culture of microvascular endothelial cells. *J Chin Microc* 2002; **6**(1): 59-61.

[8] Meimoun P, Czitrom D. Coronary microvascular dysfunction and aortic stenosis: an update. *Ann Cardiol Angeiol* Sep 9 2014.

[9] Kono Y, Fukuda S, Hanatani A, Nakanishi K, Otsuka K, Taguchi H, et al. Remote ischemic conditioning improves coronary microcirculation in healthy subjects and patients with heart failure. *Drug Des Dev Ther* 2014; **8**: 1175-1181.

[10] Van Buren DJ, Tibbs TL. Lifestyle interventions to reduce diabetes and cardiovascular disease risk among children. *Curr Diab Rep* 2014; **14**(12): 557.

[11] Cohen HV, Gurung S, Quek SY, Subramanian G, Abbas A. Blood clots. Good when you're bleeding; bad when you're not bleeding hypercoagulability and thrombophilia: overview and case report. *J N J Dent Assoc* 2014; **85**(3): 17-20.

[12] Rangarajan S, Jiménez-Yuste V, Santagostino E. Adult haemophilia A patients with inhibitors: successful immune tolerance induction with a single FVIII/VWF product. *Haemophilia* 2014; **20**(6): e414-e417.

[13] Ammollo CT, Semeraro F, Xu J, Esmon NL, Esmon CT. Extracellular histones increase plasma thrombin generation by impairing thrombomodulin-dependent protein C activation. *J Thromb Haemost* 2011; **9**(9): 1795-1803.

[14] Sun Y, Xi D, Ding W, Wang F, Zhou H, Ning Q. Soluble FGL2, a novel effector molecule of activated hepatic stellate cells, regulates T-cell function in cirrhotic patients with hepatocellular carcinoma. *Hepatol Int* 2014; **8**(4): 567-575.

[15] Wang L, Yang C, Xu M, Hu M, Wang X, Zhu T. The role of soluble fibrinogen-like protein 2 in transplantation: protection or damage. *Transplantation* 2014; **97**(12): 1201-1206.

[16] Pépin D, Paradis F, Perez-Iratxeta C, Picketts DJ, Vanderhyden BC. The imitation switch ATPase Snf2l is required for superovulation and regulates Fgl2 in differentiating mouse granulosa cells. *Biol Reprod* 2013; **88**(6): 142.

[17] Yuwaraj S, Ding J, Liu M, Marsden PA, Levy GA. Genomic characterization, localization, and functional expression of FGL2, the human gene encoding fibroleukin: a novel human procoagulant. *Genomics* 2001; **71**(3): 330-338.

[18] Clark DA, Ding JW, Chaouat G, Coulam CB, August C, Levy GA. The emerging role of immunoregulation of fibrinogen-related procoagulant Fgl2 in the success or spontaneous abortion of early pregnancy in mice and humans. *Am J Reprod Immunol* 1999; **42**(1): 37-43.

[19] Xie J. *The mechanism and clinical implication of rat coronary microthrombosis induced by sodium laurate*. Huazhong Science & Technology University; 2004.

[20] Kader KN, Moore LR, Saul JM, Zborowski M, Ziats NP, Bellamkonda RV. Isolation and purification of canine adipose microvascular endothelial cells. *Microvasc Res* 2001; **61**(2): 220-226.

[21] Horai Y, Furukawa K, Iwata S, Ohizumi Y. Changes in pH increase perfusion pressure of coronary arteries in the rat. *J Pharmacol Sci* 2005; **97**(3): 400-407.

[22] de Juan-Sanz J, Nuñez E, Zafra F, Berrocal M, Corbacho I, Ibáñez I, et al. Presynaptic control of glycine transporter 2 (GlyT2) by physical and functional association with plasma membrane Ca^{2+}-ATPase (PMCA) and Na^{+}-Ca^{2+} exchanger (NCX). *J Biol Chem* 2014; **289**: 34308-34324.

[23] Kamimura D, Ohtani T, Sakata Y, Mano T, Takeda Y, Tamaki S, et al. Ca^{2+} entry mode of Na^{+}/Ca^{2+} exchanger as a new therapeutic target for heart failure with preserved ejection fraction. *Eur Heart J* 2012; **33**(11): 1408-1416.